

REGENERATION OF TRANSGENIC *CARICA PAPAYA* AND BIOCHEMICAL ANALYSIS
OF *ARABIDOPSIS THALIANA* CONTAINING THE STILBENE SYNTHASE GENE FOR
RESISTANCE TO *PHYTOPHTHORA PALMIVORA*

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAI'I AT MĀNOA IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

MOLECULAR BIOSCIENCES AND BIOENGINEERING

DECEMBER 2016

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Keywords: papaya, stilbene synthase, resveratrol, piceid

ACKNOWLEDGEMENTS

I owe the products of this thesis to a number of individuals who have helped me immensely during the course of this project.

First and foremost, I would like to extend my sincere gratitude to my thesis advisor, Dr. David Christopher, without whom this project would not have been possible. I am deeply appreciative of his support, guidance and mentorship. I am also very thankful for my committee members, Drs. Jon-Paul Bingham and Richard Manshardt, for their critical feedback, counsel, and support.

I am very grateful for the help that I have received over the course of this project from members of the Christopher lab. Dr. Luzminda Carlos-Hilario was extremely helpful in the beginning stages of this project through her guidance and knowledge, as well as by teaching tissue culture techniques. Drs. Chris Yuen and Brad Porter, as well as Kristie Matsumoto, have been invaluable in their advice and support. I would also like to thank those who have assisted with tissue culture, including Tyler Smith, Alec Sodeani, and Christyn Wong. I am deeply grateful for Dr. Jon-Paul Bingham and his help regarding HPLC and mass spectrometry work.

I also wish to express my appreciation for the funding that has supported this project by the USDA CRSEES and Hawai'i Department of Agriculture.

ABSTRACT

Papaya (*Carica papaya* L.) is an important tropical fruit crop due to its productivity and high nutrient content. Production of papaya is diminished by infection by the oomycete pathogen *Phytophthora palmivora*, which causes root, stem and fruit rot. To increase resistance to this important pathogen, papaya was previously engineered with the *VstI* gene from grapevine, which codes for the enzyme stilbene synthase, under the control of 4 different native papaya promoters. Stilbene synthase catalyzes the biosynthesis of the antimicrobial compound resveratrol. In this study, papaya were regenerated from somatic embryos to normal papaya plants using auxins 2, 4-dichlorophenoxyacetic acid and indole 3-butyric acid, as well as the cytokinin 6-benzylaminopurine. Additionally, *Arabidopsis thaliana* plants that were previously transformed with the same genetic constructs were used as heterologous models to investigate phenotypic and metabolic effects of ectopic *VstI* expression and biosynthesis of the resveratrol derivative piceid.

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ABBREVIATIONS

BAP – 6-benzylaminopurine

CaMV35S – Cauliflower Mosaic Virus 35S promoter

Cp or *Cp* – *Carica papaya*

Cp9 – Promoter for gene encoding a cationic peroxidase enzyme in papaya

Cp29 – Promoter for gene encoding a β -1,3 glucanase enzyme in papaya

Cp35 – Promoter for gene encoding a ferulate 5-hydroxylase enzyme in papaya

Cp45 – Promoter for gene encoding a hypersensitive response protein in papaya

RP-HPLC – Reverse phase - high performance liquid chromatography

IBA – Indole-3-butyric acid

JA – Jasmonic acid

LS – Linsmaier and Skoog medium

MeJA – Methyl jasmonate

MS – Murashige and Skoog medium

NAA – 1-Naphthalene acetic acid

PGR – Plant growth regulator

P. palmivora – *Phytophthora palmivora*

PRSV – Papaya Ringspot Virus

RT-PCR – Reverse transcriptase – polymerase chain reaction

ROS – Reactive oxygen species

SA – Salicylic acid

Vst1 – Gene encoding stilbene synthase from grapevine

CHAPTER I: INTRODUCTION AND BACKGROUND

Papaya and Growth Constraints

Papaya (*Carica papaya* L.) is an important tropical fruit crop, especially in developing countries, due to its productivity and high nutrient content. Grown in approximately 60 countries, it ranked third in global production in 2012 among tropical fruits (11.22 million metric tons produced), accounting for approximately 15 percent of the tropical fruit market, excluding bananas (Evans and Ballen, 2012). In Hawai'i, papaya sales were estimated at 23.5 million pounds in 2014, valued at \$11.3 million (NASS 2015).

Papaya is a perennial, dicotyledonous herbaceous tree that grows up to 10 m tall. The trees produce large, fleshy fruits that are rich sources of A and B vitamins, as well as the commercially important enzyme papain. Papaya trees can be classified as either male, female, or hermaphrodite based on the type of inflorescence produced. Male flowers lack functional pistils, while the female flowers possess large bulbous ovaries with pistils and no stamens. Hermaphroditic trees produce bisexual inflorescences and are the dominant type in commercial cultivation due to superior fruit quality (reviewed in Persley and Ploetz, 2003). The papaya genome consists of a relatively small 372 Mbp divided into 9 chromosomes (Ming et al., 2008). Sex is determined by a single locus, with the heterozygous condition giving rise to male and hermaphroditic plants and the homozygous recessive condition yielding female plants (Storey 1976; Manshardt 1992).

A major constraint on papaya production involves diseases from a variety of sources, including viral and fungal pathogens. The main disease currently affecting papaya in Hawai'i is caused by the oomycete pathogen *Phytophthora palmivora*. Oomycetes are phylogenetically

unrelated to fungi, yet share similar morphology and function (reviewed in Lamour et al., 2007). Over 50 *Phytophthora* species infect more than 1,000 plant species, leading to significant plant damage and financial losses (Erwin and Ribeiro, 1996). This genus includes *P. infestans*, the causal agent of the Irish Potato Famine, and *P. sojae*, a devastating soybean pathogen. In papaya, *P. palmivora* causes fruit, stem, and root rot and can result in diminished productivity and/or loss of trees, especially evident in wet conditions (Nishijima, 1994). Very little natural resistance against *Phytophthora palmivora* exists in the narrow papaya gene pool, which makes developing pathogen resistance through breeding difficult (Porter et al., 2013).

In the early 1990's, the papaya ringspot virus (PRSV) caused devastating losses in papaya crops in Hawai'i. Control of the disease was achieved by creating transgenic papaya expressing a gene encoding the PRSV coat protein, a technique that confers what is known as coat protein mediated resistance (Fitch et al., 1992). The development of these resistant papaya plants, known as SunUp (homozygous) and Rainbow (hemizygous), is largely credited with saving the papaya industry in Hawai'i. This technique demonstrates the utility of biotechnology in solving problems associated with plant disease, and as such, though this study utilizes a different technique, developing an approach using bioengineering to create papaya plants resistant to *P. palmivora* is a rational and realistic goal.

Stilbene Synthase, Resveratrol and Transgenic Plants

One option towards engineering pathogen resistance in papaya is the use of the gene encoding the enzyme stilbene synthase. Stilbene synthase (STS, EC 2.3.1.95) is a 90 kDa homodimer which catalyzes the formation of the antimicrobial polyphenolic compound *trans*-3,

5, 4'-trihydroxystilbene, commonly known as resveratrol (Liswidowati et al. 1991). Formation of resveratrol and its derivatives, classified as phytoalexins, *in planta* has been shown to provide wide spectrum antibacterial, antifungal and anti-oomycete properties (Adrian et al., 1997; Dercks and Creasy, 1989; reviewed in Jeandet, 2002). In addition, resveratrol has been linked to a wide range of putative health benefits in humans including increased longevity and chemopreventative, cardioprotective, neuroprotective, and antioxidant activities (Anekonda, 2006; reviewed in Baur and Sinclair, 2006; King et al., 2006; Athar et al., 2007).

Genes coding for stilbene synthase have been found naturally occurring in a select group of generally unrelated species including peanut (*Arachis hypogaea* L.), pine (*Pinus strobus* L.), sorghum (*Sorghum bicolor* L.), and Japanese knotweed (*Polygonum cuspidatum* Sieb. et Zucc.), along with grapevine (*Vitis vinifera* L.) (Parage et al., 2012). Papaya naturally lacks a gene for stilbene synthase, though it does, like most plants, possess a gene for the related enzyme chalcone synthase (CHS, EC 2.3.1.74). Chalcone synthase is thought to have given rise to stilbene synthase independently in each of the above listed cases (Tropf et al. 1994). Chalcone synthase is highly similar to stilbene synthase in structure and sequence, however the two are functionally very different (Schröder and Schröder 1990). While both enzymes utilize the same endogenous precursor molecules, namely one moiety of 4-coumaroyl-CoA and three moieties of malonyl-CoA (Schröder et al. 1988), stilbene synthase catalyzes the production of the stilbene parent molecule, resveratrol, while chalcone synthase catalyzes the production of naringenin chalcone, a flavonoid precursor (Figure 1). Due to the presence of an appreciably large pool of endogenous precursors, the introduction of the stilbene synthase enzyme is sufficient for stimulating resveratrol production.

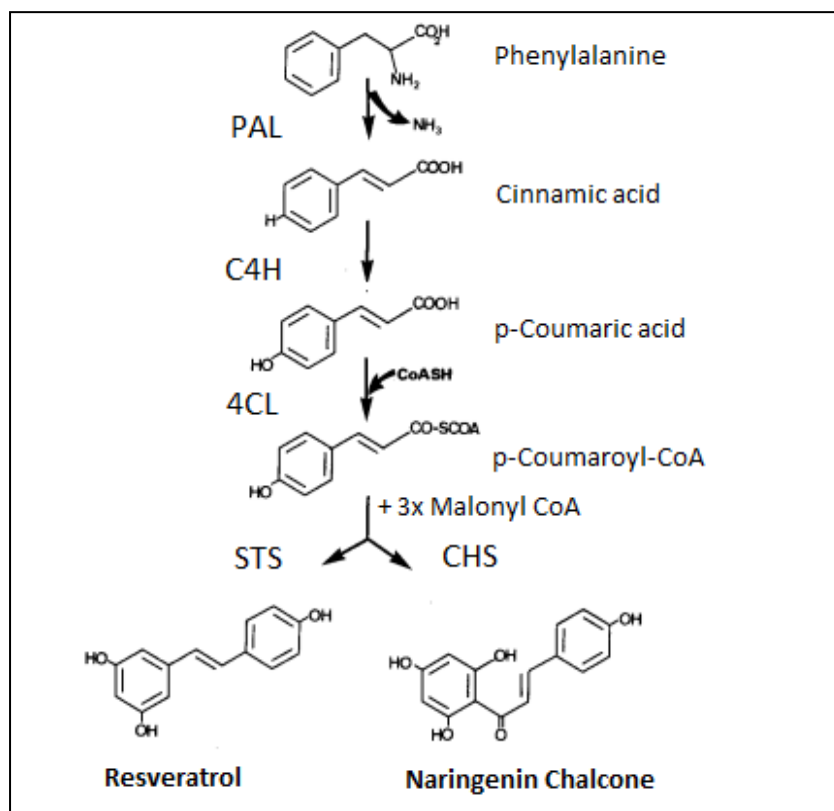


Figure 1: Phenylalanine/polymalonate pathway leading to stilbene or chalcone synthesis. PAL: Phenylalanine ammonia lyase; C4H: Cinnamate 4-hydroxylase; 4CL: Coumarate: CoA ligase; STS: Stilbene synthase; CHS: Chalcone synthase. Figure adapted from Jeandet et al. 2002.

In plants, resveratrol has been found to exist in its native form and as various chemical derivatives (Figure 2). Due to the presence of the double bond connecting the A and B rings of the resveratrol backbone, *trans* and *cis* conformations can potentially be formed. Although stilbenoids exhibiting the *cis*-conformation do exist, their occurrence is more limited than those with the *trans* conformation due to instability brought on by steric hindrance between the two rings (Chong et al., 2009). Accordingly, all further discussion will be primarily concerned with the *trans* conformation. From the resveratrol backbone, various chemical substitutions can be introduced *in planta* to create stilbene derivatives with differing antimicrobial properties. Common derivatives include piceid (glycosylated), pterostilbene (doubly methylated), and the

viniferins (resveratrol dimers) (Figure 2). Piceid (*trans*-resveratrol-3-*O*- β -D-glucopyranoside) was found to be the dominant stilbene compound formed in the transgenic papaya on this project, and thus this study focused on piceid content (data not shown).

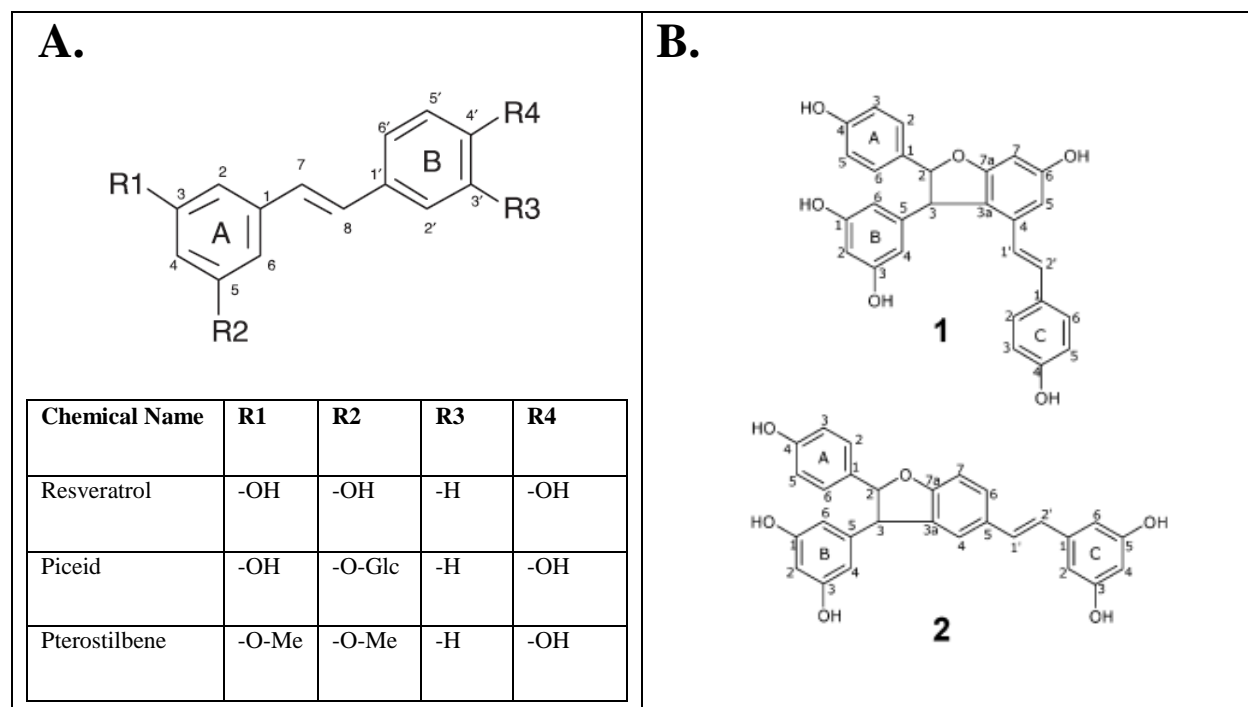


Figure 2. A.) Structures of *trans*-resveratrol and select derivatives. Glc: Glucosyl, Me: Methyl
B.) Structure of ϵ -viniferin (1) and δ -viniferin (2). Adapted from Delaunois et al. 2009 and Pezet et al. 2003.

While many studies have confirmed the antimicrobial efficacy (Adrian et al., 1997; Sobolev et al., 2011; Chalal et al., 2014), the exact mechanism for the antimicrobial properties of resveratrol and its derivatives has yet to be fully elucidated. However, recent studies have shed light on the interaction between resveratrol and various microbial pathogens. In bacteria, Hwang and Lim (2015) showed that resveratrol induces DNA fragmentation and prevents the formation of the bacterial Z-ring structure (a structure critical for prokaryote cell division) by inhibiting *FtsZ* expression, which encodes constituent FtsZ Z-ring proteins, thereby preventing cell division in *E. coli*. Also in *E. coli*, resveratrol was found to induce localized oxidative damage to the cell

membrane (Subramanian et al., 2014). In fungi (*Candida albicans*), resveratrol decreases mitochondrial membrane potential along with promoting cytochrome C release and caspase pathway activation, leading to apoptosis (Lee and Lee, 2015). There is limited information concerning the interaction between resveratrol and oomycete pathogens, however evidence suggests that it disrupts protein-protein interactions involved in microtubule formation in *Phytophthora megasperma*, which may explain findings suggesting that resveratrol disrupts normal structuring and hyphal branching (Porchewski et al., 2001).

Transgenic Plants Expressing STS Genes

Due to the antimicrobial properties of resveratrol, the stilbene synthase gene has been of interest in the field of crop protection. Many transgenic plant species transformed with genes coding for stilbene synthase have demonstrated increased resistance to a variety of pathogens (Table 1). The first species to be transformed with the stilbene synthase gene was tobacco, which showed an increased resistance to *Botrytis cinerea* (Hain et al., 1993). Subsequent work with wheat and barley also produced resistance to this pathogen (Leckband and Lorz, 1998; Serazetdinova et al., 2005). From the table, it is evident that stilbene concentrations and the type of pathogen resistance vary widely depending on the genetic construct used (both gene and promoter) and the plant system being transformed.

Table 1. Select studies involving various transgenic plant species expressing stilbene synthase and the biological effects of transformation. *Vst1*+35S-4 fold is a combination of promoter elements from both the native *Vst1* promoter and the 35S promoter. Adapted from Delaunois et al., 2009.

Plant Species	Gene	Promoter	Biochemical Species	Stilbene Concentration (µg/g FW)	Biological Activity	Reference
Tobacco (<i>Nicotiana tabacum</i> L.)	<i>Vst1</i> and <i>Vst2</i>	<i>Vst1</i>	<i>trans</i> -Resveratrol	400	Resistance to <i>Botrytis cinerea</i>	Hain et al., 1993
	Chimeric <i>STS</i> gene	<i>CaMV35S</i>	Resveratrol	50-290	Altered flower morphology, male sterility	Fischer et al., 1997
Wheat (<i>Triticum aestivum</i> L.)	<i>Vst1</i>	<i>Vst1</i> +35S-4 fold	-	-	Resistance to <i>Botrytis cinerea</i>	Leckband and Lorz, 1998
	<i>Vst1</i> and <i>Vst2</i>	<i>Vst1</i> +35S-4 fold	-	35-190	Resistance to <i>Puccinia recondita</i> and <i>Stagonospora nodorum</i>	Serazetdinova et al., 2005
Barley (<i>Hordeum vulgare</i> L.)	<i>Vst1</i>	<i>Vst1</i> +35S-4 fold	-	-	Resistance to <i>Botrytis cinerea</i>	Leckband and Lorz, 1998
Alfalfa (<i>Medicago sativa</i> L.)	<i>AhRS</i>	<i>CaMV35S</i>	<i>trans</i> -Piceid	0.5-20	Resistance to <i>Phoma medicaginis</i>	Hipskind and Paiva, 2000
<i>Arabidopsis thaliana</i> L.	<i>PcRS</i>	<i>CaMV35S</i>	<i>trans</i> -Piceid	93-183	Resistance to <i>Colletotrichum higginsianum</i>	Liu et al., 2011
Papaya (<i>Carica papaya</i> L.)	<i>Vst1</i>	<i>Vst1</i>	<i>trans</i> -Piceid	54	Resistance to <i>Phytophthora palmivora</i>	Zhu et al., 2004

The choice of which promoter to use for genetic engineering is of particular value, as the promoter largely controls when the gene is expressed and in what tissues, as well as the strength of expression. In this case, the balance between the health of the plant and the destruction of the microbe is of critical importance. The constitutive *CaMV35S* promoter typically brings high stilbene production, yet has been associated with adverse phenotypic effects such as male sterility, potentially a result of the depletion of endogenous precursors (Fischer et al., 1997). The vast majority of studies involving transgenic plants transformed with the stilbene synthase gene have used either the *CaMV35S* promoter or the endogenous grapevine *Vst1* promoter. Tissue specific and inducible promoters are more useful in targeting expression to tissues that need it,

and meanwhile reducing the depletion of endogenous precursors due to overexpression when and where high expression of the transgene is not needed.

Transgenic Papaya Expressing Stilbene Synthase

Since traditional papaya breeding has had very limited success against *P. palmivora*, an approach using transgenic techniques is an attractive option to limit damage caused by this pathogen. A previous study performed by Zhu et al. (2004) demonstrated that ectopic expression of the grape stilbene synthase gene driven by the endogenous grape *Vst1* promoter in transgenic papaya resulted in an approximate 65% reduction in disease symptoms over WT controls when challenged with *P. palmivora* inoculum. However, the native grape promoter failed to provide early enough expression of the gene to protect against the pathogen during the most critical stages of infection in papaya. In addition, no basal (pre-infection) expression was observed, which would confer protection for the plant before infection starts. Though the results of the study were encouraging, there remains room for improvement and optimization, which is the basis of the present study.

Our lab has recently produced transgenic papaya somatic embryos and *Arabidopsis thaliana* lines that express the gene *Vst1*, which was isolated from grapevine (*Vitis vinifera*). The *Vst1* gene has been fused to four different native papaya promoters and introduced into *Arabidopsis* and papaya. The long-term goal is to provide enhanced tissue-regulated and pathogen-induced expression in the papaya plants. The genes for these promoters were identified by Porter et al. (2008), and were found to be pathogen induced, with high levels of expression mainly localized to the roots and stems (Porter et al. 2009). The four genes encode a cationic

peroxidase (*Cp9*), a β -1,3-glucanase (*Cp29*), a ferulate 5-hydroxylase enzyme (*Cp35*), and a hypersensitive-induced response protein (*Cp45*). Three of these genes, *Cp29*, *Cp35*, and *Cp45*, were upregulated by *P. palmivora* infection, while the fourth, *Cp9*, was downregulated (Porter et al., 2009). Despite this downregulation, which the authors speculate is a result of the action of pathogen effectors, *Cp9* was included in this study due to high basal expression in roots, the primary infection site for *P. palmivora*. These native papaya promoters are ideal for localizing expression of the *VstI* gene to roots and stems of papaya, tissues that are highly susceptible to *P. palmivora* infection. Table 2 below displays properties of the native papaya promoters used. The *CaMV35S* promoter was also fused to *VstI* and is used as a positive control. Carlos-Hilario et al. (2015) isolated and characterized the promoters from these four genes and conducted the genetic engineering. Unfortunately, no transgenic lines containing the *Cp29:VstI* construct were recovered in papaya, though all other lines have been regenerated or are in the process of regeneration.

Cp9 – Cationic Peroxidase

The *Cp9* gene was isolated by Porter et al. (2009) and codes for a Class III cationic peroxidase enzyme. Peroxidases are present in all land plants (Passardi et al., 2004), and comprise a large, multigenic family of enzymes that perform a wide variety of functions. In general, peroxidases modify the concentration of reactive oxygen species (ROS) such as H_2O_2 and OH^\bullet by both their hydroxylic and peroxidative cycles, by oxidation of various substrates in the presence of hydrogen peroxide, and also by producing hydroxyl radicals (Liszkay et al., 2003; reviewed in Passardi et al., 2005). The function of peroxidases includes lignification and suberization of the cell wall, loosening of the cell wall during growth and elongation, auxin

catabolism, and detoxifying chemical contaminants (reviewed in Passardi et al., 2005). In response to pathogenic recognition by the host plant, many isoforms of peroxidases have been found to be upregulated by a wide range of fungal, bacterial, and viral pathogens, and are responsible for strengthening the cell wall by cross-linking and generating toxic levels of ROS to defend against pathogen infection (reviewed in Hiraga et al., 2001). In papaya, *Cp9* was found to be downregulated in roots in response to challenge with *P. palmivora*, a consequence that the authors speculate is a result of pathogen effectors (Porter et al., 2009). In spite of this downregulation, *Cp9* has a high expression mainly localized to the roots, and thus the *Cp9* promoter remains an attractive promoter option.

Cp29 – β -1,3 Glucanase

The *Cp29* encodes a β -1,3 glucanase enzyme that is part of the pathogenesis related PR-2 family of proteins. The β -1,3 glucanase enzyme hydrolyzes β -1,3 glycosidic bonds in glucan polymers, which are highly abundant in the cell walls of some pathogens, most notably the oomycetes. During a defense response, regulation of expression of PR-2 proteins has been shown to be salicylic acid (SA) responsive, with mRNA levels increasing approximately 20-fold in response to exogenous SA (Uknes et al., 1992). Glucan oligosaccharides have been shown to be potent elicitors of defense responses, including production of phytoalexins (Darvill and Albershiem, 1984), thus it is thought that these glucan degrading enzymes serve both to reduce the integrity of the pathogen cell wall and to release elicitors leading to heightened host defense response. In papaya, *Cp29* was found to be upregulated in roots, where expression is mainly localized, by approximately 15-fold in response to challenge by *P. palmivora* (Porter et al., 2009), thus the *Cp29* promoter is an attractive option for conferring optimal expression patterns.

Cp35 – Ferulate-5-Hydroxylase

The *Cp35* gene encodes the cytochrome P450 enzyme ferulate-5-hydroxylase (F5H), which is implicated in the biosynthesis of lignin. Specifically, F5H catalyzes the production of 5-hydroxyferulic acid, which is then converted to sinapic acid and subsequently syringyl (S) lignin units (Ruegger et al., 1999). In *Arabidopsis*, S lignin is highly tissue specific, and the ratio of S lignin seems to be directly related to F5H gene expression levels (Meyer et al., 1998). The production of lignin imparts mechanical strength to plant tissues and provides a physical barrier to potential pathogens. The expression of F5H is modulated in *Camptotheca acuminata* mostly by abiotic stresses such as wounding, application of the plant hormones abscisic acid (ABA), ethylene, and exogenous H₂O₂ (Kim et al., 2005). As hydrogen peroxide is also implicated in roles concerning situations involving biotic stress, expression of F5H may also be regulated by biotic stresses to some extent, as evidenced in papaya. In papaya roots, where expression is mainly localized, F5H gene expression was upregulated approximately 5-fold in response to challenge with *P. palmivora* (Porter et al., 2009), thus the *Cp35* promoter is another attractive option for conferring optimal expression patterns.

Cp45 – Hypersensitive Induced Response Protein

The *Cp45* gene encodes a hypersensitive induced response (HIR) protein that is implicated in the process of localized cell death caused by the activation of the hypersensitive response. The *Cp45* protein is highly similar in sequence to an HIR protein, CaHIR1, in pepper (*Capsicum annuum*) (Porter et al., 2009), which has been shown to cause localized cell death and

kinase-dependent transcriptional upregulation of a suite of PR genes (Jung and Hwang, 2007). In general, the localized cell death caused by the hypersensitive response is thought to limit the spread of pathogenic growth, especially that of biotrophic pathogens, and represents an active defense process by the host plant (reviewed in Greenberg, 1997). Indeed, in *Arabidopsis* ectopically expressing the rice HIR protein OsHIR1, the transgenic plants showed a lower titer of pathogen (Pst DC3000) as compared to untransformed controls (Zhou et al., 2010). In both papaya roots and leaves, expression of *Cp45* was upregulated by approximately 5-fold in response to challenge with *P. palmivora* (Porter et al., 2009), and thus the *Cp45* promoter is another attractive option for conferring optimal expression patterns.

Table 2. Selected native papaya genes from which promoters were isolated and used for driving the *VstI* transgene. ORF: Open reading frame. Adapted from Porter et al., 2009.

ORF	Protein Homology	Expression Change	Function
<i>Cp9</i>	Cationic Peroxidase	Down	PR: Cell wall reinforcement, ROS production
<i>Cp29</i>	β -1,3-glucanase	Up	PR: Degrades β -glucan chains, releases glucan oligosaccharide elicitors
<i>Cp35</i>	Ferulate 5-hydroxylase	Up	Abiotic stress; Lignin and secondary metabolite synthesis
<i>Cp45</i>	Hypersensitive response protein	Up	PR: Involved in programmed cell death

CHAPTER II: RATIONALE, HYPOTHESES, AND OBJECTIVES

Rationale and Hypotheses

This study was conducted in conjunction with a past study (Carlos-Hilario, 2015) to regenerate previously transformed papaya cultures containing the *VstI* gene from somatic embryos to fully formed papaya plants. The grape *VstI* gene codes for stilbene synthase, which catalyzes the biosynthesis of resveratrol from common endogenous precursor molecules (Figure 2). Resveratrol has proven antimicrobial properties *in vitro* and *in vivo* and thus is a valuable compound for increasing resistance to the potent oomycete pathogen *Phytophthora palmivora*.

Transformation of plants with the stilbene synthase gene, *VstI*, has been previously shown to increase plant resistance to a variety of pathogens (see Table 1). The choice of promoter to drive the transgene is critical for achieving the desired expression patterns. The *CaMV35S* promoter has been used in many studies (reviewed in Delaunoy et al., 2009), which confers a high level of constitutive expression in all tissues. However, overexpression of the stilbene synthase gene can lead to detrimental substrate competition between STS and CHS, and can lead to morphological abnormalities (Fischer et al., 1997), therefore a more regulated promoter is desirable to mitigate these effects. Zhu et al. (2004) previously showed that papaya transformed with *VstI* exhibit up to 65% increased resistance to infection by *P. palmivora*. However, the regulated promoter used to drive the transgene in this previous study, the native *VstI* inducible promoter from grape, provided expression patterns that were likely not optimal on a temporal and spatial basis. No basal expression of the *VstI* gene was detected in young or older plants, which would confer an important preformed defense mechanism. Additionally,

VstI exhibited no localized expression to any tissue type. However, based on past success, there is a high likelihood that the transformation of the papaya in the current study will confer increased pathogen resistance as well. Additionally, the native papaya promoters used in this study were chosen based on expression principally localized in the roots and stems, where initial infection is likely to occur, with an intermediate level of basal expression and relatively high inducibility.

Though the appropriate papaya promoter-*VstI* genetic constructs have already been created and transformed into the papaya and *Arabidopsis* genomes, the verification of altered *VstI* expression and its putative expression patterns have yet to be thoroughly investigated. Quantification of both *VstI* mRNA levels and the final yield of resveratrol and its derivatives in plant tissues are necessary to characterize the levels of the gene expression and the resulting biochemical output of compounds. However, the transgenic papaya somatic embryos must be regenerated to form stable, mature papaya plants in order for this to be carried out in the papaya plants. Based on past successes, I hypothesized that these papaya lines can be regenerated from somatic embryos using a combination of plant growth regulators.

In the meantime, *Arabidopsis* plants have previously been transformed with identical genetic constructs and serve as heterologous models for the papaya. Basal piceid levels in two of the four promoter:*VstI* lines have been previously characterized. Based on indications from transgenic papaya somatic embryos, I hypothesize that the *Cp35:VstI* lines will contain a relatively low amount of piceid, while the *Cp45:VstI* lines will contain an intermediate piceid content. Resveratrol and its derivatives have been shown to be more effective at preventing fungal growth at higher concentrations (Chalal et al., 2014), thus it would be beneficial to propagate those genetic lines that express *VstI* at higher levels in key tissues for pathogen

resistance studies in the future, yet balance that high expression with the health of the plant. These analyses will help direct the further study of the transgenic papaya.

Both salicylic acid and jasmonic acid are major plant hormones involved in responding to pathogen attack. Exogenous application of these hormones activates the expression of their respective defense pathways and gene expression. By measuring the change in expression patterns of the Cp promoter:*VstI* constructs in Arabidopsis in response to the two hormones, valuable information can be gathered as to which types of defense mechanisms the promoter constructs are controlled by and the strength of the response. I hypothesize that expression levels of at least some of the Cp promoter:*VstI* constructs will be elevated in response to both salicylic acid and jasmonic acid, based on the idea that the promoters used are involved in defense response pathways in papaya.

As an effect of the introduction of the transgene, certain other related biochemical processes may be affected. Anthocyanins belong to the flavonoid family of secondary metabolites and may be affected in certain situations such as exposure to blue light. I hypothesize that the anthocyanin levels will be affected as a result of the introduction of the transgene in response to blue light. In addition, I hypothesize that the levels of piceid will be affected following blue light exposure as well, based on direct competition for precursor molecules by stilbene synthase and chalcone synthase.

Objectives

The overall objectives of this project are to regenerate the transgenic papaya Cp promoter:*VstI* lines from somatic embryos. However, since regeneration of papaya plants was slow, another emphasis was placed on testing a chemical induction system in the Arabidopsis Cp promoter:*VstI* lines, with subsequent measurement of *VstI* mRNA levels to determine changes in transgene expression during a defense response caused by exogenous salicylic acid.

- i. **Regenerate transgenic papaya transformed with Cp promoter:*VstI* genetic constructs into mature plants.**
 - a. Use the synthetic auxin 2,4-dichlorophenoxyacetic acid to induce formation of somatic embryos from globular clusters of transgenic papaya cells.
 - b. Use the plant hormone 6- benzylaminopurine to develop normal mature shoots from germinated somatic embryos and callus tissue.
 - c. Determine the optimal concentrations of the plant hormone indole-3-butyric acid for initiating normal mature roots in sterile conditions.
 - d. Transfer normal mature plants to soil to propagate.
- ii. **Characterize the gene expression and biochemical changes in Cp promoter:*VstI* Arabidopsis lines in response to the defense response elicitors salicylic acid and methyl jasmonate, as well as blue light, an abiotic elicitor**
 - a. Design primers to amplify the positive control genes *PR1* and *PDF1.2*

- b. Determine change in relative abundance of *PR1* and *PDF1.2* transcripts between Arabidopsis samples treated with SA and MeJA and untreated samples, as compared to the reference gene *Actin2*, using semi-quantitative RT-PCR techniques.
- c. Determine change in relative abundance of *Vst1* transcripts between Arabidopsis samples treated with SA and MeJA and untreated samples, as compared to the reference gene *Actin2*, using semi-quantitative RT-PCR techniques.
- d. Determine basal levels of piceid in transgenic Arabidopsis lines *Cp35:Vst1* and *Cp45:Vst1*
- e. Determine biochemical changes in endogenous anthocyanin levels and ectopic piceid levels as a response to blue light using spectrophotometric and RP-HPLC techniques.

CHAPTER III: REGENERATION OF PAPAYA SOMATIC EMBRYOS

Introduction

Papaya Somatic Embryogenesis and Regeneration

As a result of the transformation process, our transgenic papaya require regeneration from somatic embryos. The regeneration of papaya somatic embryos into mature plants has been a subject of study for the past 30 years. Somatic embryogenesis in papaya primarily proceeds through formation of undifferentiated callus tissue, from which somatic embryos develop.

Generally, somatic embryogenesis in plants involves inducing the potential to form an embryo in tissues that are not normally embryogenic (i.e. in somatic cells). Somatic embryogenesis is morphologically similar to normal zygotic embryogenesis, though the molecular pathways behind both processes may not be similar. Somatic embryos go through the same morphological developmental stages as do zygotic embryos, namely the globular, heart, torpedo and cotyledonary stages. However, somatic embryos do not have the associated suspensor cells as do zygotic embryos, suggesting that perhaps culture conditions and plant growth regulators (PGRs) take over the role of signaling from the suspensor cells (reviewed in Dodeman et al., 1997). Since the first discovery of somatic embryogenesis in carrot, it has been discovered that most plants have the ability to convert at least a certain population of cells into somatic embryos (reviewed in von Arnold et al., 2002).

For a plant somatic cell to become embryogenic, it needs first to exchange its current set of gene expression patterns for one that is more favorable to embryo formation. How this

change is effected is not entirely clear, though it is thought that exogenous application of auxins changes the rate of DNA methylation, therefore shifting gene expression to create conditions more similar to an embryonic state (LoSchiavo, 1989). However, this mechanism may not be entirely responsible for the embryogenic transition in all plants, as treatment with DNA methylation inhibitors, such as 5-azacytidine, have not always halted the process (Leljak-Levanic, 2004). Auxins have also been shown to promote competent cell division and proliferation into embryogenic cell clusters, known as proembryogenic masses (PEM's), though subsequent differentiation and development after this seems to be inhibited by auxin. After transfer to PGR-free medium, the development of the somatic embryos starts and the embryo proceeds into the subsequent heart, torpedo, and cotyledonary stages of development (Komamine et al., 1992; reviewed in von Arnold et al., 2002).

Once the somatic embryos reach the cotyledonary stage, other PGR's, such as cytokinins or gibberellins, may be required to stimulate further growth, development, and elongation. Cytokinins are usually associated with the formation and growth of shoot systems in the developing plant. Upon treatment with cytokinins, shoots develop out of the clumps of cells containing the somatic embryos. The mature shoots are then usually excised from excess tissue and transferred into rooting media containing auxin to stimulate root formation and growth. The molecular mechanisms involved in shoot and root growth seem to be highly dependent on a number of factors, including most notably the type of plant species, as evidenced by the high number of diverse protocols for regenerating various plant species.

A variety of tissues have been used as explants to provide the material for somatic embryo generation in papaya, including hypocotyl, axillary bud, root, ovule, and zygotic embryo (reviewed in Dhekney et al., 2016). In this study, hypocotyl sections of papaya seedlings were

used as explants, and papaya plantlets were regenerated from the resulting callus tissue with timed application of auxins and cytokinins to the growth media.

Materials and Methods

Media Preparation and Culture Conditions

All media containing different chemical constituents were prepared in a similar fashion. The pH of all media was adjusted to pH 5.6-5.8 using 1 M potassium hydroxide (KOH) or hydrochloric acid (HCl) as needed. Plant growth regulators were added to the media before autoclaving via fresh 100 mg/L stock solutions. To solidify all media, 3.2 g/L Gelrite® was added, and media was autoclaved at 121°C and 15 psi. All cultures were kept in 25 ±2°C conditions supplied with ~35 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ cool white fluorescent light on a 16 hr. day/ 8 hr. night schedule.

Genetic Transformation

Genetic transformation of papaya with the *Cp9*, *Cp29*, *Cp35*, or *Cp45* promoter coupled to the *VstI* gene was carried out prior to this study by Carlos-Hilario (2015). Briefly, papaya cv. Kapoho seedlings were germinated on sterile semisolid media, and the hypocotyls were sectioned into 1 mm discs and sown onto semisolid media containing 10 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) to induce callus formation. The resulting callus tissue was

suspended in liquid $\frac{1}{2}$ X MS media and transformed by co-cultivation with *Agrobacterium tumefaciens* containing the Cp promoter:*VstI* constructs and a hygromycin selectable marker gene. Putative transformants were selected by transfer onto semisolid selection medium containing 50 μ g/mL hygromycin B.

Induction of Somatic Embryos

Previously transformed papaya callus tissue containing the *VstI* gene coupled to one of four Cp promoters was placed in 100 x 15 mm sterile petri plates containing semisolid $\frac{1}{2}$ X Murashige and Skoog (MS) basal media with vitamins (Phytotechnology Labs, Kansas, USA) containing 50 mg/L *myo*-inositol, 0.4 g/L glutamine, 70 g/L sucrose and 3 mg/L 2,4-D to induce somatic embryogenesis. Cultures were kept in the dark. Calli were subcultured every 3-4 weeks and brown or dead tissue was discarded, while friable somatic embryos were transferred to $\frac{1}{2}$ X MS basal media with vitamins supplemented with 30 g/L sucrose and 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (MES) for further development.

Shoot Induction from Germinated Somatic Embryos

Once germination occurred, somatic embryos were transferred to $\frac{1}{2}$ X MS semisolid media with vitamins supplemented with 30 g/L sucrose, 0.5 g/L MES, 0.3 mg/L 6-benzylaminopurine (BAP) and 0.02 mg/L naphthaleneacetic acid (NAA) at pH 5.6-5.8 to induce shoot formation. Cultures were maintained by subculturing in the same media every 3-4 weeks. Cultures that became too large to fit into petri plates were switched to Magenta® GA-7 polycarbonate boxes containing the same media. To reduce hormonal stress, healthy shoots with

leaves were excised from clumps and sown onto PGR-free $\frac{1}{2}$ X MS semisolid media with vitamins supplemented with 5 g/L sucrose and 0.5 g/L MES, with either 0 or 200 mg/L activated charcoal.

Root Induction from Developed Shoots

Healthy shoots measuring more than 2 cm tall were transferred to root induction media containing $\frac{1}{2}$ X MS semisolid media with vitamins supplemented with 5 g/L sucrose, 0.5 g/L MES and 0.5 mg/L indole-3-butyric acid (IBA). Cultures were kept in this rooting media for either 3 or 7 days, either in the light or in the dark, and after were transferred to $\frac{1}{2}$ X MS semisolid media with vitamins supplemented with 5 g/L sucrose and 0.5 g/L MES. After 3 weeks, the number of roots or root initials were recorded within each treatment (with or without activated charcoal (an adsorbent) prior to auxin treatment, in light or dark during auxin treatment, 3 or 7 days in root induction medium) to determine rooting percentages within each treatment.

Hardening and Transfer to Ambient Conditions

Plants with fully formed root and shoot systems were transferred to non-sterile 4" square pots containing a 1:1 mixture of soil:vermiculite moistened with water containing $\frac{1}{2}$ X MS salts and vitamins. The pots were wrapped in plastic wrap and were slowly unwrapped slightly each day for 2 weeks in order for the plants to acclimatize to ambient conditions. However, after less than optimal results with this method, plants with fully formed root and shoot systems were thereafter transferred to 1" circular pots containing non-sterile Sta-Green® horticulture-grade

vermiculite moistened with water containing ½X MS salts and vitamins. These pots were contained in Magenta® boxes to maintain a humid environment, and to allow the plants to grow accustomed to soil-like conditions and to further develop root systems. To accustom the fully formed plants to the ambient environment, plants showing healthy root and shoot systems and expanded leaves were first subject to decreased humidity by opening the lid of the container approximately 1-2 mm, with ambient conditions at 25° C and approximately 65% RH. Once the plants were accustomed to the ambient humidity (approximately 1 week), they were transferred to pots containing 1:1 mixture of soil:vermiculite.

Results

Somatic Embryogenesis

Transgenic callus tissue in the globular stage responded to the 2,4-D treatment by forming friable callus that then developed into torpedo-shaped somatic embryos after transfer to auxin-free media (Figure 3). The lines containing the *Cp9:Vst1* construct formed somatic embryos at a much faster rate than any of the other lines, due to unknown causes.

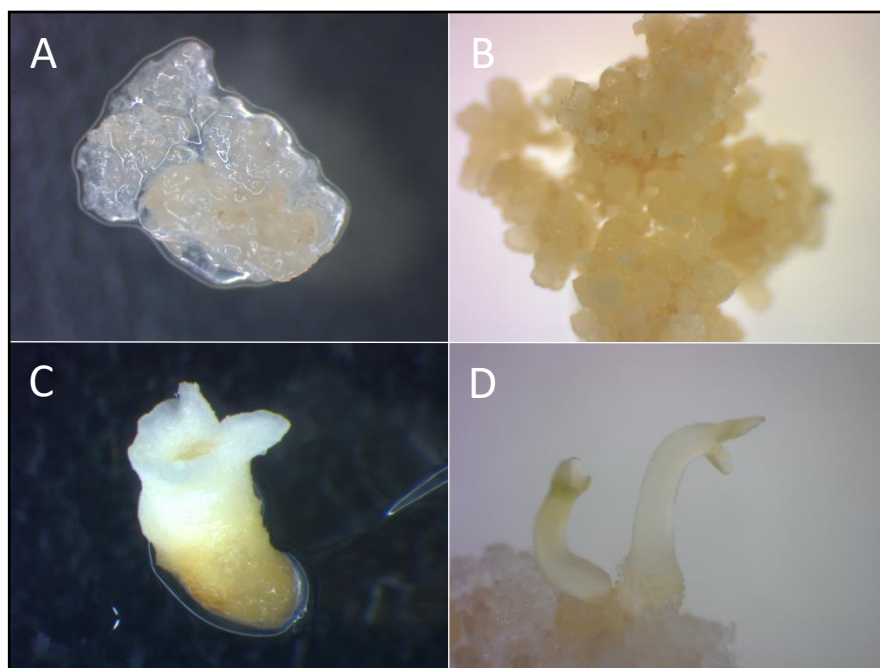


Figure 3: Depiction of papaya somatic embryos at various stages of development.
A.) Globular stage/early callus. B.) Developing into friable callus. C) Torpedo stage. D.) Cotyledonary stage.

Somatic Embryo Germination and Shoot Induction

Somatic embryos germinated into clumps of tissue with shoot forming potential. Cytokinin treatment with BAP at a concentration of 1.0 mg/L produced clumps that formed many shoots, but also resulted in a relatively high amount of toxicity, as evidenced by brown and dead tissue. These shoots also frequently produced auxiliary shoots coming off the main hypocotyl. The shoots formed from this treatment also seemed to take a longer than normal amount of time to produce roots in response to the auxin treatment, compared to other reports (data not shown; Yang and Ye, 1992; Wu et al., 2012). BAP and NAA at a concentration of 0.3 mg/L and 0.02 mg/L, respectively, was sufficient to form shoots, though the shoots formed were not as

frequently occurring or as elongated as those produced after treatment with 1.0 mg/L BAP. Lines containing the *Cp9:Vst1* construct responded much more robustly and quickly to treatment with cytokinins. Shoots with leaves were excised from clumps and transferred to PGR-free ½X MS media with or without 200 mg/L activated charcoal to allow for healthy growth and time for excess cytokinin to be degraded or leached into the media and adsorbed by the activated charcoal. At the time of writing, the *Cp35:Vst1* and *Cp45:Vst1* lines have not progressed beyond this stage due to slower (than the *Cp9:Vst* lines) development.

Rooting Induction

Roots were induced in healthy, fully formed shoots by treatment with the auxin IBA in the growth media. Only those plants containing the *Cp9:Vst1* construct reached this point in development. Somatic embryos containing the other three genetic constructs were slow to regenerate and, at the time of this writing, have not yet reached the proper developmental stage necessary for root induction. Those shoots that formed root initials or roots in response to treatment 4 weeks after the treatment was concluded were tallied, and rooting percentages are displayed in Table 3. In general, rooting treatment was also characterized by mild to severe leaf abscission, with more severe abscission seemingly correlated with higher auxin concentrations (data not shown). Rooting treatment for 7 days in the dark with 0.5 mg/L IBA yielded the highest amount of plants with roots after 3 weeks post treatment. However, this treatment also caused the highest amount of leaf abscission.

Table 3. Rooting treatments and percent of plants in which rooting was induced. n= number of plants in each treatment.

Treatment concentration	Treatment duration (days)	Light condition	Activated Charcoal concentration (mg/L)	Rooting %	n
0.3 mg/L IBA	7	light	0	14.3	16
0.3 mg/L IBA	7	light	200	17.9	32
0.4 mg/L IBA	7	light	0	7.1	14
0.4 mg/L IBA	7	light	200	0	9
0.5 mg/L IBA	3	light	0	0	18
0.5 mg/L IBA	3	light	200	5.9	17
0.5 mg/L IBA	7	dark	0	26.3	38

Hardening and Transfer to Ambient Conditions

Plants with fully formed root and shoot systems were transferred to 4” square pots containing non-sterile 1:1 soil:vermiculite and wrapped in plastic wrap. All four plants that were hardened this way died within two weeks of transfer. Therefore adjustments were made to transfer rooted plants into non-sterile vermiculite in Magenta boxes, and slowly adjust them to ambient conditions by opening the container by a small amount (1-2 mm) every day. As of the time of this writing, ten papaya plants, all containing the *Cp9:Vst1* construct, have been transplanted into vermiculite and are currently thriving.

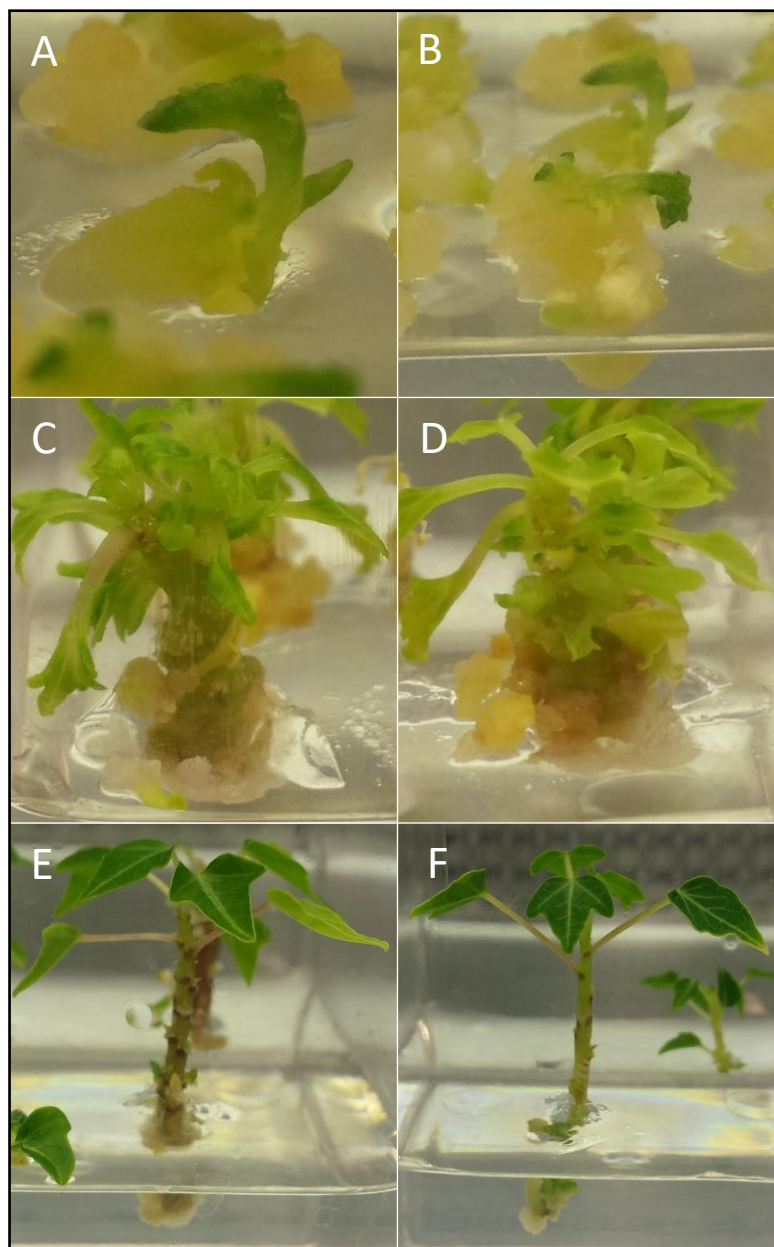


Figure 4. Depiction of papaya plantlets at various stages of shoot development. Beginnings of organogenesis and leaf formation (A) and (B), some elongation and formation of organized shoot apical meristems (C) and (D), and mature shoots with developed leaves but lacking roots (E) and (F).

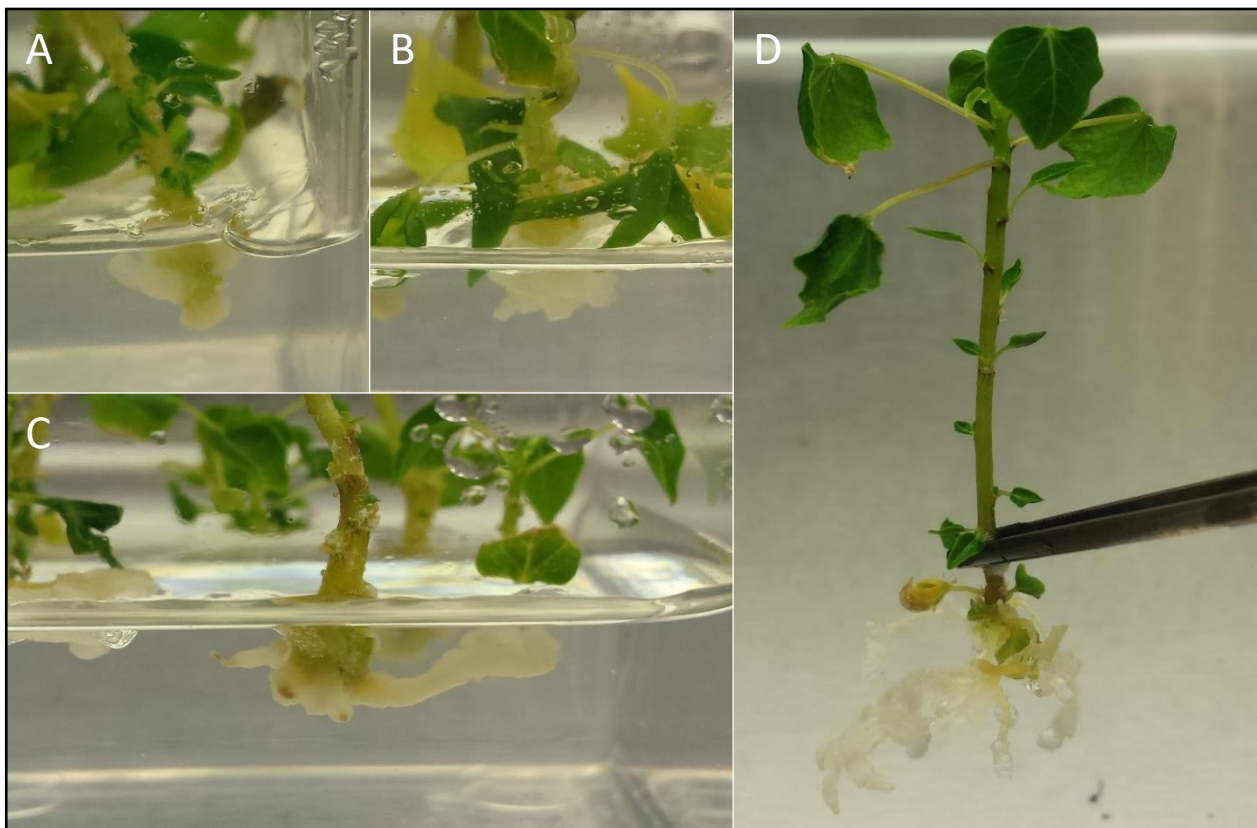


Figure 5. Depiction of papaya plantlets at various stages of root formation. Development of root initials (A) and (B), elongation of roots (C), and a plant with a mature root system ready to go into soil conditions (D).

Discussion

Somatic Embryogenesis

In response to treatment with the auxin 2, 4-D, somatic embryos formed from callus tissue, albeit at a low frequency. Somatic embryos were most apparent on the tops of callus clumps, where later stage embryos were frequently observed. This is consistent with the fact that auxin is inhibitory for further development after somatic embryo formation (Komamine et al.,

1992). The auxin concentration is presumably much lower on the top of the clump as compared to the surface of the media, so somatic embryos located on top have a much higher chance of developing more normally than embryos located closer to the media surface.

Shoot Induction

Treatment with the cytokinin BAP produced clumps of cells from which shoots developed. Most shoots had defined apical meristems, although elongation of these shoots was variable. Treatment with relatively high concentration of BAP (1.0 mg/L) caused prolific growth of shoots from clumps, though dead and brown tissue formed on the underside of these clumps, suggesting some degree of cytokinin toxicity. Though BAP at this concentration did produce a multitude of shoots, this concentration may be overly high, as some loss of apical dominance was observed as evidenced by frequent axillary shoots coming off the main hypocotyl. Additionally, these shoots were extremely difficult to root (see Root induction, below), which may be explained by an overabundance of cytokinin in shoot tissues after treatment.

Root Induction

Inducing root formation in healthy shoots was the most difficult part of the regeneration process. Roots formed at a low rate, even after treatment with IBA. The treatment that produced the highest percentage of roots formed was 0.5 mg/L IBA for 7 days in the dark. Roots also formed at a low rate in untreated (i.e. no auxin) plantlets, suggesting that some endogenous processes also can account for root formation. However, the rate of this occurrence was much too low to be a useful and viable option for regeneration. Rooting percentages following

treatment with IBA were much lower than other reports. For example, groups have reported rooting percentages as high as 90% (Wu et al., 2012). The protocol used in the present study was derived from Yang and Ye (1992), who reported 92% rooting after treatment with 0.5 mg/L IBA for 7 days. This high rooting percentage could not be reached using the same conditions in this study, as the best percentage of rooting was approximately 26%. Rooting percentages associated with similar treatments (i.e. IBA treatment at 0.5 – 2 mg/L) vary widely in the literature, and it seems that what worked best in one case may not be optimal in all other cases.

Some diversity in procedures may account for these differences. The cultivar used as initial explants most likely has a large effect on the outcome of regeneration and the efficacy of treatments, as different cultivars may respond differently to the same treatment. The type of tissue used for explants likely also has a large effect on regeneration efficiency, with different tissues requiring differing conditions to regenerate. For example, shoot tips can be regenerated directly to form mature papaya plants without going through the callus stage, thereby reducing the requirement for exogenous auxin to induce somatic embryos. (Wu et al., 2012). Age of cultures is also likely a factor, as older cultures can exhibit somaclonal variation and lose totipotency and the ability to develop into functional plants (reviewed in Jain 2001).

Rooting treatment is usually carried out using IBA. Although other auxins may be used, IBA is more efficient than NAA at producing roots (De Winaar, 1988). Despite consistent usage, the concentration of IBA is difficult to keep uniform, as it seems to be both somewhat heat labile and light sensitive. IBA concentrations are reduced by approximately 20% following autoclaving. Additionally, IBA is degraded by about 80% after only 3 days exposed to light in agar medium containing MS salts (Nissen and Sutter, 1990), a fact that may explain why the best root induction occurred in plantlets kept in the dark, as a lower percentage of auxin is degraded

in the dark, leading to higher bioavailability. Following this, it is likely that there are significant discrepancies between the IBA concentrations reported and the concentrations that are actually experienced by the plant, though in some cases such as light exposure, this degradation would be difficult to avoid. To minimize variation, IBA stock solutions should be prepared fresh and filter sterilized to avoid as much chance of degradation as possible.

Hardening/Acclimatization

Transfer to soil and the presumable drop in humidity (even with the plastic wrap) were seemingly difficult conditions for the small plants to grow accustomed to, as all of the plants that were hardened in this manner died within the first 2 weeks. All of these plants displayed drooping of leaves and relatively rapid leaf abscission, presumably symptoms related to rapid changes in humidity. Therefore, adjustments were made to harden the plants at a slightly slower pace using Magenta boxes. The Magenta boxes keep a constant humidity better than pots wrapped in plastic wrap, so that the change in humidity was not as drastic after the initial transfer, and humidity could be more gradually lowered to ambient levels after that. The first two plantlets that were transferred into vermiculite were done so into a sterile environment, while those following were transferred into non-sterile vermiculite. All plantlets thrived after transfer to vermiculite regardless of the sterile conditions or not, implying that sterile conditions are not necessary at this point.

Conclusion

The transgenic papaya callus tissue and the regeneration protocols used in this study were sufficient to regenerate whole, mature transgenic papaya plants. The transition from suspension derived transgenic papaya cells to somatic embryos was effected by treatment with the synthetic auxin 2,4-dichlorophenoxyacetic acid. Maturing somatic embryos were induced to form shoots by treatment with the cytokinin benzylaminopurine, while roots were formed at a relatively low rate after treatment with the auxin indole-3-butyric acid. The adjusted protocol for hardening the plantlets had a high degree of success using a process of slow adjustment to ambient conditions. The *35S:Vst1*, *Cp35:Vst1*, and *Cp45:Vst1* lines have so far regenerated at a slow rate, and none of these lines have mature shoots. However, those papaya lines containing the *Cp9:Vst1* construct regenerated at a much faster rate than any of the other lines, due to unknown factors. A total of ten *Cp9:Vst1* containing papaya plants have been regenerated and planted into soil-like conditions, with a high potential for many more to be regenerated given some more time.

CHAPTER IV: TRANSGENIC *ARABIDOPSIS THALIANA* AND INVESTIGATION OF PROMOTER INDUCIBILITY

Introduction

Arabidopsis thaliana plants were transformed previously with identical genetic constructs as those used in papaya by Carlos-Hilario (2015). In a separate part of this thesis, these *Arabidopsis* plants were used to investigate the transcriptional regulation of the Cp promoter: *VstI* genetic constructs as expressed in a heterologous model system. Due to the slow development and regeneration time of transgenic papaya, transgenic *Arabidopsis* plants were transformed alongside papaya. The fast growing model plant serves as an expedient heterologous system for quick and facile analyses of promoter expression patterns (Hilario et al., 2015). It can also be used to determine the transcriptional and biochemical responses to various stimuli, such as the defense response elicitors salicylic acid and methyl jasmonate, as well as abiotic elicitors such as blue light.

Salicylic Acid

Salicylic acid (SA) is a key plant hormone involved in responding to pathogen invasion, and is involved in initiating systemic acquired resistance (SAR) in plant systems (Delaney et al., 1994). SAR is a signal transduction pathway that can be initiated by both pathogen attack and herbivory by insects, and can confer broad-spectrum resistance to a variety of organisms, including bacterial, viral and fungal pathogens (reviewed in Sticher et al., 1997). The induction of SAR activates the expression of a suite of defense related genes, which includes genes coding for the pathogenesis related (PR) proteins PR-1 (unknown function), PR-2 (β 1,3-glucanase), and

peroxidases (Smith et al., 1991; Uknes et al., 1993). The *PR-1* gene is typically used as an indicator of SA pathway activation, as it is normally highly upregulated after SA signaling (Palva et al., 1994). The underlying molecular mechanism of SA pathway activation has been suggested to be due to SA binding the aptly named salicylic acid binding protein (SABP), therefore inhibiting the normal catalase activity of SABP. The inhibition of SABP causes H₂O₂ levels to rise, subsequently activating downstream transcriptional changes leading to a defense response (Chen et al., 1993). This increase in hydrogen peroxide levels is thought to activate the hypersensitive response, which involves a large set of biochemical and transcriptional changes culminating in localized programmed cell death (reviewed in Lamb and Dixon, 1997). Studies have shown that application of exogenous SA is sufficient to induce the biochemical and transcriptional changes that are associated with SAR (Chen et al., 1993; Senaratna et al., 2000). Therefore, exogenously applied SA can activate a defense response and activate SAR in the absence of any pathogen, a fact that is leveraged by the agricultural sector with the application of the stable synthetic SA analog benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) (Gorlach et al., 1996), commercially sold under the trade name Actigard®.

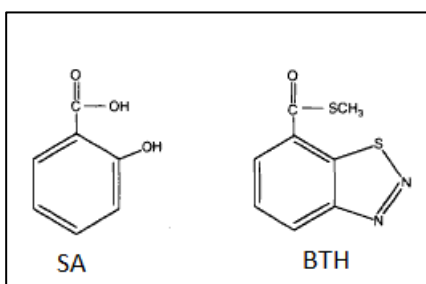


Figure 6. Structures of salicylic acid (SA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), an SA analog.

Methyl Jasmonate

Along with salicylic acid, jasmonic acid (JA) is a major plant hormone involved in plant defense against pathogens. Perception of JA activates a seemingly separate set of defense related genes from those activated by salicylic acid (Penninckx et al., 1996; Thomma et al., 1998). Sensitivity to JA is controlled by the *COI1* locus, and *coi1* mutants are unresponsive to jasmonic acid and its analogs, methyl jasmonate (MeJA) and coronatine, and are also male sterile (Feys et al., 1994). JA can be conjugated to several different amino acids, and the isoleucine-JA conjugate has been suggested to be the active form (Staswick and Tiryaki, 2004). Perception of jasmonic acid initiates expression of a suite of resistance genes including genes coding for PDF1.2 (plant defensin protein), PR-3 (chitinase), and PR-4 (hevein-like protein) (van Loon et al., 2006). The *PDF1.2* gene is typically used as an indicator of JA pathway activation. The genes activated by JA are generally more suitable for defense against a necrotizing pathogen, as programmed cell death would be counterproductive to plant defense during necrotrophic infection (Glazebrook, 2005). SA is generally antagonistic to the JA pathway, as SA has been found to inhibit JA biosynthesis and action (Spoel et al., 2003). Due to the inducing properties of MeJA even in the absence of a pathogen, it is highly suitable for studying the gene expression of the Cp promoter: *VstI* constructs when defense related pathways are activated.

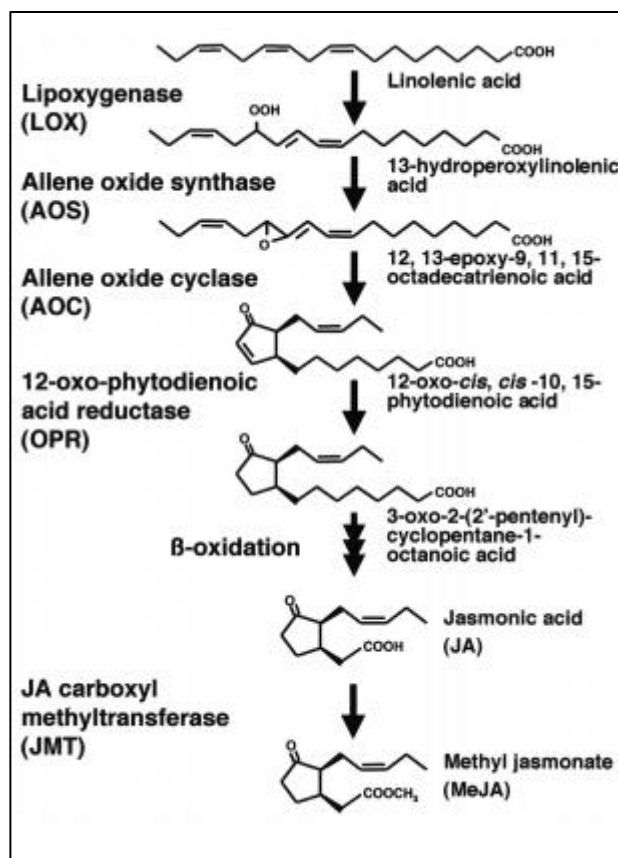


Figure 7. Jasmonic Acid synthesis pathway. Adapted from Sasaki et al. 2001.

Blue Light Response

Plants are exposed to radiation from the sun as a consequence of exposure to sunlight during photosynthesis. In many cases, this radiation can be highly energetic, such as UV rays, or excessive (i.e. high intensity), potentially resulting in damage to the plant. The biosynthesis of certain molecules that absorb UV and excess radiation and accumulate in the vacuoles of epidermal cells can be viewed as one of the ways that plants deal with potentially damaging radiation (reviewed in Carletti et al, 2014; reviewed in Demmig-Adams and Adams, 1992).

Flavonoids are a large class of secondary metabolites characterized by two variously substituted aromatic rings connected by a C3 unit. Flavonoids absorb light strongly in the UV

range, and in response to exposure to UV light, plants generally activate the transcription of genes responsible for flavonoid biosynthesis in order to protect themselves from damage from UV radiation (reviewed in Koes et al., 1993). Anthocyanins are polyphenolic pigment molecules belonging to the flavonoid family, and are involved in a range of functions including pigmentation/pollinator attraction, antioxidant activities, and resistance to insect attack (reviewed in Kong et al., 2003), as well as mitigating stress from excess light energy (Smillie and Hetherington, 1999).

Though not as potentially damaging as UV radiation to plants, blue light ($\lambda = 450\text{-}495$ nm) has been shown to activate a similar but separate pathway to induce many of the same responses as those that are produced in response to UV radiation. In *Arabidopsis* seedlings, exposure to both UV and blue light causes increased transcript accumulation of many of the genes involved in flavonoid and anthocyanin biosynthesis, including phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), and dihydroflavonol reductase (*DFR*) (Kubasek et al., 1992). Of these, *PAL* and *CHS* are involved in the synthesis of naringenin chalcone (see Figure 1), while *CHI* converts naringenin chalcone to the flavanone naringenin, and *DFR* is involved in the multi-step process of converting dihydroflavonols into anthocyanins. Upregulation of these biosynthetic genes in response to blue light results in increased flavonoid content, including anthocyanins.

Excess visible light energy can result in photoinhibition of Photosystem II, leading to decreased photosynthesis (Christopher and Mullet, 1994; reviewed in Demmig-Adams and Adams, 1992). Anthocyanins have been implicated in mitigating damage associated with excess light energy (Smillie and Hetherington, 1999; Havaux and Kloppstech, 2001), which may explain the purpose of increased synthesis in response to blue light. Anthocyanins are

synthesized in multiple steps from the precursor naringenin chalcone, which is the direct product of chalcone synthase (Tsao 2010). As stated previously, chalcone synthase and stilbene synthase are direct competitors for precursor molecules. Therefore, an increase in the activity of one enzyme, and associated increase in downstream metabolites, may bring about a decrease in the levels of products and intermediates in the other pathway. Experiments described involving induction by blue light are designed to begin to test this hypothesis.

In this study, transgenic *Arabidopsis* plants were subjected to treatment with the chemical defense response elicitors salicylic acid and methyl jasmonate, as well as abiotic elicitation in response to blue light. Treatment with these elicitors shows how transgenic *Arabidopsis* might regulate the *VstI* transgene in response to both biotic and abiotic stresses. By extension, using *Arabidopsis* as a model, these data could point to how transgenic papaya regulate the *VstI* transgene in response to pathogen challenge and abiotic stresses, and show how other endogenous processes are affected as well.

Materials and Methods

Transgenic Arabidopsis

Arabidopsis (Col-0) were previously transformed (Carlos-Hilario et al., 2015) with one of four native papaya promoters coupled to the *VstI* gene, each with at least three independent lines. Transformation was carried out via the floral dip method using *Agrobacterium*

tumefaciens bearing the recombinant genetic constructs. This study used lines containing the *Cp9:VstI*, *Cp29:VstI*, *Cp35:VstI* and *Cp45:VstI* constructs.

SA and MeJA Experiments

Plant Growth and Treatments

Two independent transgenic lines from each of the four Cp promoter:*VstI* construct were used, and each independent line consisted of 5 biological replicates. Seeds were sterilized with a 10% sodium hypochlorite and 0.1% Tween 20 solution and, following stratification at 4°C for 2 days, were sown in sterile 125mL Erlenmeyer flasks containing 50 mL ½X liquid Linsmaier and Skoog (LS) media containing 30 g/L sucrose and 1g/L MES (Phytotechnology Labs, Kansas, USA), 40 seeds per flask. After 2 weeks of growth, the media was replaced with either another 50 mL ½X LS media (control), or the same media supplemented with 250 µM salicylic acid (SA) or 100 µM methyl jasmonate (MeJA). Samples were harvested at 24 hrs. after treatment and were manually homogenized under liquid N₂ using a mortar and pestle. Samples were stored at -80°C until use.

Semi-Quantitative RT-PCR

RNA was extracted from homogenized plant tissue using the NucleoSpin RNA Plant extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. mRNA from 2 µg total RNA was converted to cDNA using M-MLV Reverse Transcriptase (RT; Promega, Madison, WI, USA) and oligo d(T)15 primers, per the manufacturer's protocol. Single

stranded cDNA was converted to double stranded DNA fragments using Bio-X-Act Short Mix (Bioline, London, UK) and primers for *VstI* (Forward – 5'-GAGGAAATTAGAAACGCTCAACGTGCCAAGGGTC-3', Reverse – 5'-GAACGCTATGCAGCACAACGGTCTCAATGGTC-3') and the reference gene *Actin2* (Forward – 5'-TTGCAGGAGATGATGCTCCAGG-3', Reverse – 5'-CATTCCCACAAACGAGGGCTGG-3'). Additionally, primers for genes known to be upregulated by SA and MeJA, *PR-1* and *PDF1.2*, respectively, were used as positive controls for induction (*PR-1* Forward – 5'-GTAGCTCTTGTAGGTGCTCTTGTTC-3', Reverse – 5'-CGTTCACATAATTCCCACGAGGATC-3'; *PDF1.2* Forward – 5'-ATGGCTAAGTTTGCTTCCATCATCACCC-3', Reverse – 5'-GTGCTGGGAAGACATAGTTGCATGATCC-3'). Negative control samples from the RT phase containing no RT enzyme were also included in the PCR reaction to detect potential amplification of contaminating genomic DNA. PCR conditions were as follows: 95°C for 1 min., (95°C for 30 sec., 60°C for 30 sec., 72°C for 2 min.), 72°C for 10min. Conditions in parentheses were cycled for varying numbers of cycles. The number of PCR cycles for each primer set was adjusted in order to ensure products were imaged in the logarithmic range of amplification to prevent saturation. 10 µL of PCR products were run out on a 0.8% TAE agarose gel stained with 0.01% GelRed (Biotium Inc., Hayward, CA, USA) and imaged using a Kodak 4000 Multimodal Image Station. Positive induction control and *VstI* band intensities were normalized to those of *Actin2* using Kodak Molecular Imaging software (Kodak Co., Rochester, NY, USA).

Verification of RNA Integrity

Purified RNA samples were run out on a denaturing gel to verify that the RNA was free from any degradation that might skew band intensities during semi-quantitative RT-PCR. RNA loading buffer consisting of formamide (53%), 10X MOPS buffer (11%), formaldehyde (13%), 50% glycerol (14%), 10% bromophenol blue (1%), and nuclease free water (8%, percent volume) was mixed with 2 µg total RNA from each sample in a 2:1 buffer:sample (v:v) ratio. Samples were loaded and electrophoresed on a 1.2% agarose gel containing 1.5% formaldehyde, 10% 10X MOPS buffer (200 mM MOPS (N-morpholino propanesulfonic acid), 50 mM sodium acetate, and 10 mM EDTA at pH 7, autoclaved), and 0.01% GelRed. All chemical components and materials used were RNase free. Gels were visualized using a Kodak 4000 Multimodal Image Station. RNA was evaluated on the basis of the integrity of the prominent 25S and 18S ribosomal subunit bands.

RP-HPLC Analysis of Basal Piceid Content

Reverse phase high performance liquid chromatography (RP-HPLC) was used to quantify the biochemical output of piceid in transgenic Arabidopsis plants as basal levels in the *Cp35:Vst1* and the *Cp45:Vst1* lines. Basal levels of the *35S:Vst1*, *Cp9:Vst1*, and *Cp29:Vst1* lines were reported previously by Hilario et al. (2015), and methods used to determine basal piceid content were the same in this study. All above ground tissues were harvested from 4 week old plants. Samples were ground under liquid nitrogen using a mortar and pestle. The amount of tissue was determined, and 2 mL of 80% ethanol was added to the ground tissue to extract the

metabolites. Samples were shaken overnight at 100 rpm at 4°C, and following this, each sample was centrifuged twice at 10,000 g, each time removing the insoluble material. The resulting supernatant was concentrated to dryness using a vacuum centrifuge. Samples were redissolved in 80 µL of 100% HPLC grade methanol and 920 µL of filtered 2% (v/v) acetic acid in ultrapure water. The resulting solution was again centrifuged twice at 10,000 g to remove any remaining insoluble material. Samples were stored at -20°C until use.

The standard resveratrol glucoside (piceid) (Sigma-Aldrich, St. Louis, MO, USA) and samples of tissue extracts containing piceid were resolved by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), which consisted of a Waters 2695 automated HPLC system interfaced with a Waters 996 Photodiode Array Detector (PDA), scanning 210-600 nm. Peaks were resolved using a Kinetex C18 column (Phenomenex; 2.6 µm; 100Å; 100 x 4.6 mm) at a flow rate of 0.8 mL/min and eluted with a linear 8%/min gradient of Solvent A (2% v/v acetic acid/Aq.) against Solvent B (2% v/v acetic acid/MeOH), with a 3 min re-equilibration period between sample injections. Resulting chromatographic profiles were extracted at 300 nm, and the target peak area integrated using Waters Empower Pro software. Standards of piceid were analyzed in triplicate, allowing for the precise establishment of piceid concentrations reported as µg/g fresh tissue weight.

Verification of Piceid Identity

Using RP-HPLC, fractions of the target peak putatively containing piceid were collected and subject to mass spectrometry to verify piceid identity. Mass spectrometry experiments were undertaken using an AB/MDS-Sciex API 3000 triple quadrupole mass spectrometer (Thornhill,

Ontario, Canada). The ESI–MS system was calibrated manually in positive mode with PPG 3000 (AB/MDS-Sciex) to achieve <5-ppm mass accuracy, as per manufacturer’s protocol. The mass spectrometry was interfaced with a PE ABI 140D Solvent Delivery System. Ultrahigh purified N₂ was delivered by a Peak Scientific generator. RP-HPLC purified fractions were injected as a 20 µL bolus, at a flow rate 25 µL/min, in a solvent of 70% MeCN/30% 0.1% v/v formic acid/Aq. Initially, full ion spectra were taken from m/z 100-600 Da to provide identification of the parent molecular mass using quadrupole-3 (Q-3).

For tandem mass spectrometry (referred to as MS/MS) experiments, collision-induced dissociation (CID) of the identified parent molecular mass was effected by bombardment with N₂. Bombardment was confined to quadrupole-2 (Q-2) with a collision cell gas thickness of 3×10^{14} atoms/cm² and a collision energy (Q-0 to Q-2 rod offset voltage) typically set at ~20–40 eV. The resulting CID (daughter ion) spectra were obtained by scanning Q-3 from m/z 100–400 Da. MS/MS data analysis was assisted with the use of Analyst Software (v1.4.1; AB/MDS-Sciex) and ACD ChemSketch (ACD/Labs). Mass spectra of the samples were compared to a commercial piceid standard (Sigma-Aldrich, St. Louis, MO, USA).

Blue Light Treatment

Growth Conditions

Arabidopsis seeds were sown in soil and subjected to three different lighting conditions. The first (Control) consisted of cool white fluorescent lights at an intensity of 30 µmol/m²*s for the entire growth period of 9 days. The second (Light Control) consisted of LED lights emitting at wavelengths 460-470 nm and at an intensity of 150 µmol/m²*s for the entire growth period.

The third treatment (Treatment) consisted of identical conditions as the second with the exception of an increase in intensity from 150 to 450 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ for two days, starting at the 6th day, and a decrease back down to 150 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ for the duration of the 9th and final day.

Anthocyanin Quantification

After the 9 day growth period, all above ground tissue was harvested, weighed and soaked in 1 mL 1% (v/v) HCl in ethanol in 1.5 mL microcentrifuge tubes. Samples were manually homogenized using a small pestle, and metabolites were extracted overnight. Samples were centrifuged to remove insoluble material, and total anthocyanin content of the prepared supernatant from each of the samples was detected using a spectrophotometer measuring at $\lambda=530$ nm and $\lambda=657$ nm. The formula $A_{530}-(0.25\cdot A_{657})$ was used to correct for chlorophyll absorbance (Rabino and Mancinelli, 1986), and results were reported in corrected A_{530} per gram fresh weight (gfw). One sample each consisting of approximately 10-20 seedlings from 3 independent lines was analyzed per Cp promoter:*VstI* construct.

Piceid Quantification

After the 9 day growth period, all above ground tissue was harvested, weighed and soaked in 1 mL 80% ethanol. Samples were manually homogenized using a small pestle, and metabolites were extracted overnight. The RP-HPLC analysis protocol was identical to the one used to determine basal piceid content.

Results

SA Experiments

Cp9:VstI lines

Based on PCR band intensities, expression of the positive control gene *PR-1* was upregulated by 10.79X and 22.82X as compared to untreated controls in lines 13 and 15, respectively. The *VstI* gene was seemingly not regulated by SA treatment, as expression was very close to the same as untreated controls, at 0.95X and 0.89X for lines 13 and 15, respectively. When the data from the two lines containing the *Cp9:VstI* construct are pooled together, the positive control expression was upregulated $17.94X \pm 2.85$ (SE) over untreated controls, and the *VstI* gene expression was relatively unchanged at $0.92X \pm 0.10$. RNA was verified as intact via RNA denaturing gel (data not shown).

Cp29:VstI lines

Based on PCR band intensities, expression of the positive control gene *PR-1* was upregulated by 7.94X and 5.81X as compared to untreated controls in lines 23 and 25, respectively. The *VstI* gene was seemingly not regulated by SA treatment, as expression was very close to the same as untreated controls, at 0.99X and 1.03X for lines 23 and 25, respectively. When the data from the two lines containing the *Cp29:VstI* construct are pooled together, the positive control expression was upregulated $6.88X \pm 0.79$ (SE) over untreated controls, and the *VstI* gene expression was relatively unchanged at $1.01X \pm 0.08$. RNA was verified as intact via RNA denaturing gel (data not shown).

Cp35:VstI lines

Based on PCR band intensities, expression of the positive control gene *PR-1* was upregulated by 7.35X and 4.29X as compared to untreated controls in lines 29 and 32, respectively. The *VstI* gene was seemingly not regulated by SA treatment, as expression was very close to the same as untreated controls, at 1.13X and 1.19X for lines 29 and 32, respectively. When the data from the two lines containing the *Cp35:VstI* construct are pooled together, the positive control expression was upregulated $5.82X \pm 0.84$ (SE) over untreated controls, and the *VstI* gene expression was relatively unchanged at $1.16X \pm 0.10$. RNA was verified as intact via RNA denaturing gel (data not shown).

Cp45:VstI lines

Based on PCR band intensities, expression of the positive control gene *PR-1* was upregulated by 50.19X and 103.15X as compared to untreated controls in lines 34 and 35, respectively. The *VstI* gene was seemingly not regulated by SA treatment, as expression was very close to the same as untreated controls, at 1.39X and 1.03X for lines 34 and 35, respectively. When the data from the two lines containing the *Cp9:VstI* construct are pooled together, the positive control expression was upregulated $76.67X \pm 19.42$ (SE) over untreated controls, and the *VstI* gene expression was relatively unchanged at $1.21X \pm 0.11$. RNA was verified as intact via RNA denaturing gel (data not shown).

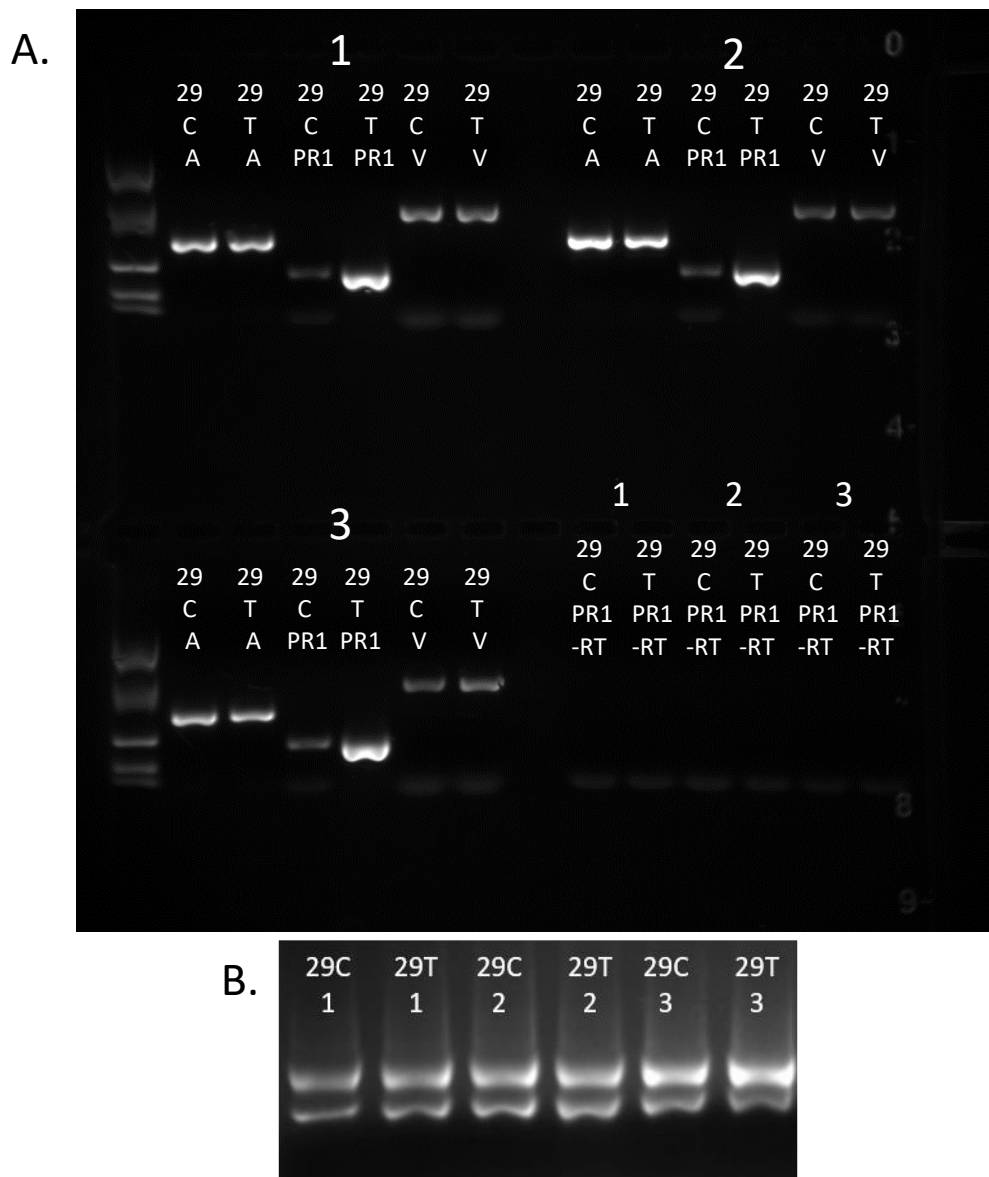


Figure 8. A.) Representative 0.8% agarose gel depicting end results of RT-PCR of *Arabidopsis* line 29 containing the *Cp35::VstI* construct treated with 250 μ M salicylic acid (SA), conducted as 3 biological replicates (1, 2, and 3). C=Untreated Control, T=SA treated sample. A=*Actin2* gene primers used, PR1=*PR-1* gene primers used, V=*VstI* gene primers used. -RT= No RT control used to show no genomic DNA contamination. B.) Representative RNA formaldehyde denaturing gel demonstrating RNA integrity by showing intact 18S and 25S ribosomal bands.

Table 4. Summary of relative *PR-1* and *Vst1* gene expression changes in response to treatment with 250 μ M salicylic acid after 24 hrs. as measured by semi-quantitative RT-PCR. Averages from each promoter construct were calculated from 2 independent transgenic lines using 5 biological replicates each.

Promoter Construct	Avg <i>PR-1</i> Regulation*	<i>PR-1</i> Std. Error	Avg. <i>Vst1</i> Regulation*	<i>Vst1</i> Std. Error
<i>Cp9:Vst1</i>	17.94	2.85	0.92	0.10
<i>Cp29:Vst1</i>	6.88	0.79	1.01	0.08
<i>Cp35:Vst1</i>	5.82	0.84	1.16	0.10
<i>Cp45:Vst1</i>	76.67	19.42	1.21	0.11

*Values are reported as fold-difference as compared to untreated controls. A value of 1 is unchanged compared to control.

Methyl Jasmonate Treatment

Treatment of Arabidopsis plants with 100 μ M methyl jasmonate resulted in variable expression of the positive control gene *PDF1.2*. The *Actin2* reference gene was relatively unchanged in all MeJA treatments. In some cases, the expression of *PDF1.2* was elevated in response to the treatment, but in other cases it was unchanged or decreased. The unreliability of the treatment to induce higher than basal levels of expression in the positive control presented a difficulty in assessing the efficacy of the treatment. Combined with unpromising results with the *Vst1* gene (i.e. no upregulation in any of the experiments), the MeJA treatment experiments were not pursued further.

Basal Piceid Content and Confirmation of Piceid Identity

Four week old *Cp35:Vst1* and *Cp45:Vst1* transgenic Arabidopsis lines were analyzed by RP-HPLC for piceid content. Piceid contained in Arabidopsis samples eluted at an equivalent retention time as that of the commercial piceid standard, approximately 7.3 min (data not shown). As compared to the piceid content in the *35S:Vst1* lines, the *Cp35:Vst1* showed a relatively low basal level of piceid, at $2.1 \pm 0.13 \mu\text{g/gfw}$. The *Cp45:Vst1* lines contained approximately twice that of the *Cp35:Vst1* lines, at $4.93 \pm 0.35 \mu\text{g/gfw}$ (Figure 9). The basal piceid content of the *35S:Vst1* ($17.3 \pm 0.77 \mu\text{g/gfw}$), *Cp9:Vst1* ($2.49 \pm 0.14 \mu\text{g/gfw}$), and *Cp29:Vst1* ($15.4 \pm 0.13 \mu\text{g/gfw}$) lines were reported previously (Hilario et al., 2015), and are included in Figure 9 for reference.

To verify the identity of the compound detected by RP-HPLC, an isolated fraction putatively containing piceid was subject to analysis by mass spectrometry, along with the commercial piceid standard for comparison. The mass spectrum generated by MS/MS analysis of a fractionated Arabidopsis sample matched with that of the piceid standard. A peak appearing at approximately 391 m/z in both spectra corresponds to the parent ion $[\text{M}+\text{H}]^+$ piceid, while other major and minor peaks correspond to fragments resulting from the breaking of various bonds within the parent ion (Figure 10).

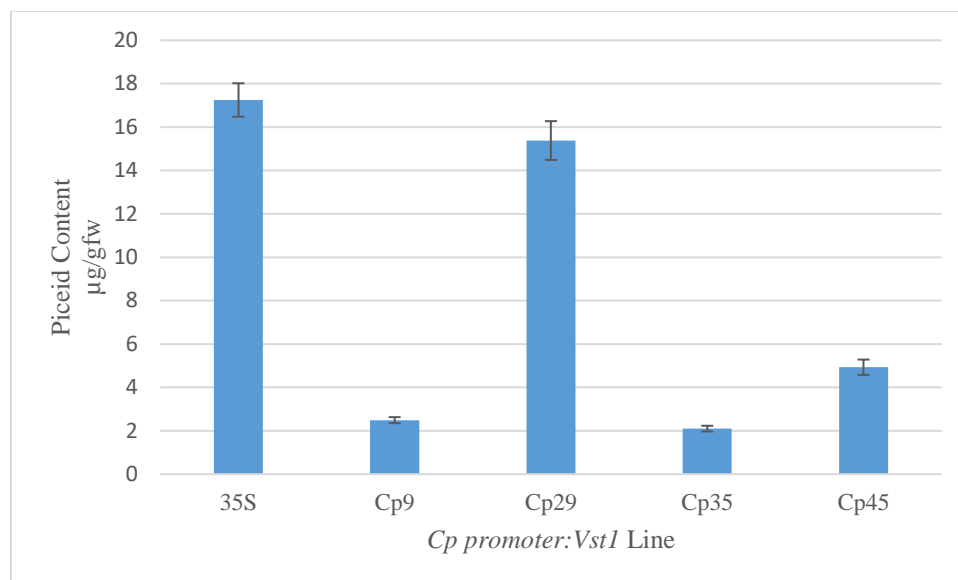


Figure 9. Basal piceid content in all five transgenic 4 week old *Arabidopsis* lines as determined by RP-HPLC. Each construct consisted of at least 3 independent lines, each with 6 replicates. Error bars represent standard error, gfw = gram fresh weight. Basal piceid content of the 35S, Cp9, and Cp29 lines were reported previously (Hilario et al., 2015) and are included here for reference.

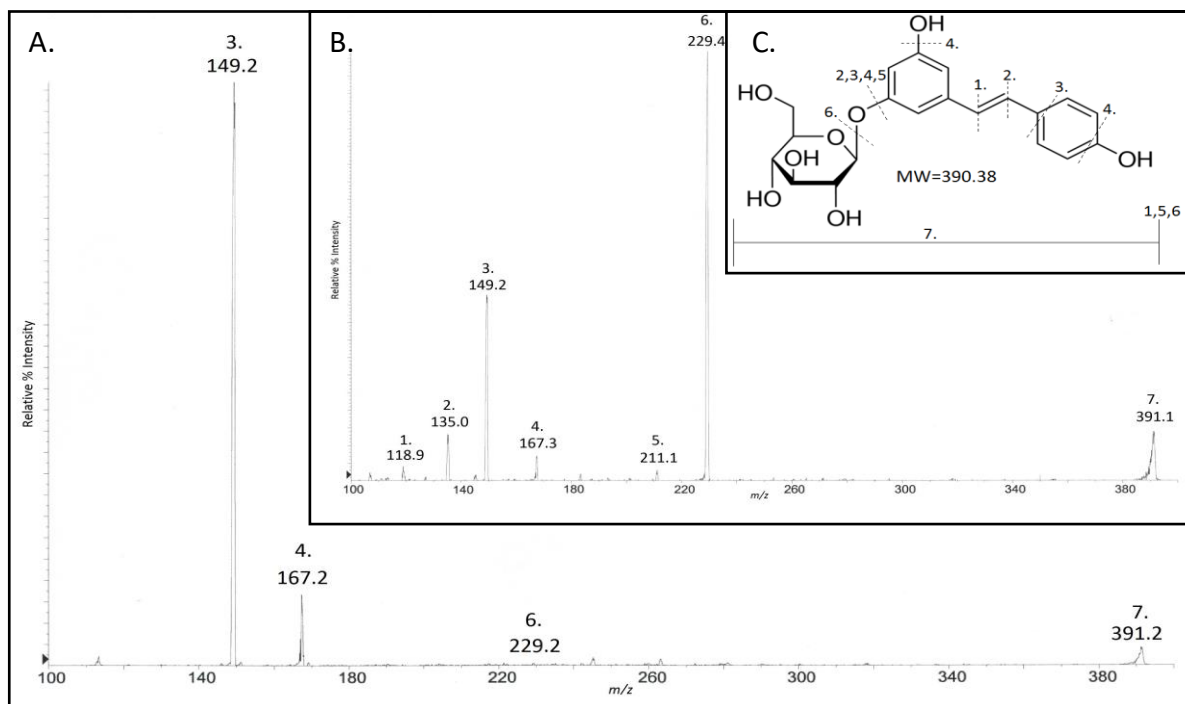


Figure 10. Mass spectrum of piceid isolated from *Arabidopsis* sample containing the *Cp29:Vst1* construct (A.) compared to the mass spectrum of the commercial piceid standard (B.). (C.) shows structure of piceid molecule and various fragments of the molecule that correspond to each numbered peak in the standard spectrum.

Blue Light Treatment

Transgenic 9-day old *Arabidopsis* plants were exposed to blue light and total anthocyanin and piceid content was measured. Spectrophotometric analysis of total anthocyanin content in the control samples revealed significant reductions in anthocyanin content in all Cp promoter:*VstI* lines, as compared to wild type (Figure 12). In the samples receiving the 450 $\mu\text{mol}/\text{m}^2\cdot\text{s}$ treatment for two days, however, anthocyanin content in all transgenic lines was either similar or higher to that of the WT control (Figure 13). Piceid content was highly variable in both the *35S:VstI* and the *Cp29:VstI* lines. The other promoter constructs exhibited relatively less variability, but in all cases, piceid content was not significantly different in plants exposed to high blue light (Treatment group) as compared to the control group (Figure 14).

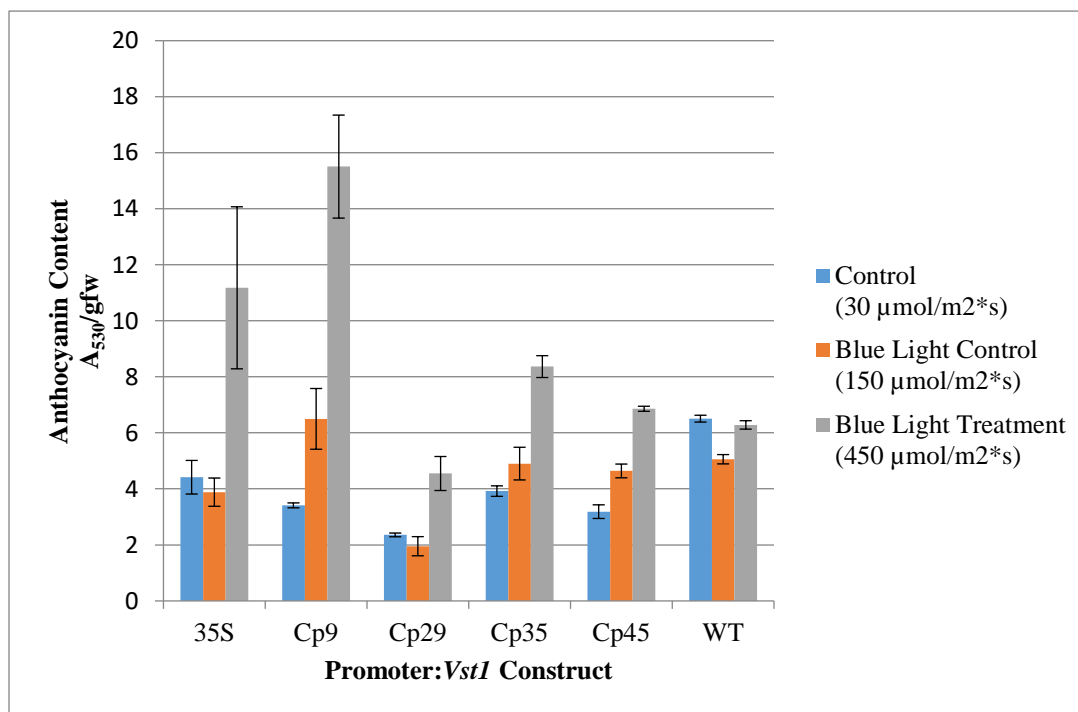


Figure 11. Anthocyanin content of representative lines of transgenic *Arabidopsis* containing each Cp promoter:*VstI* construct after treatment with either blue light (Blue Light Control and Treatment) or cool white fluorescent light (Control). Each construct consisted of 3 independent lines each, except *Cp45*, which had two. Error bars represent standard error, gfw = gram fresh weight.

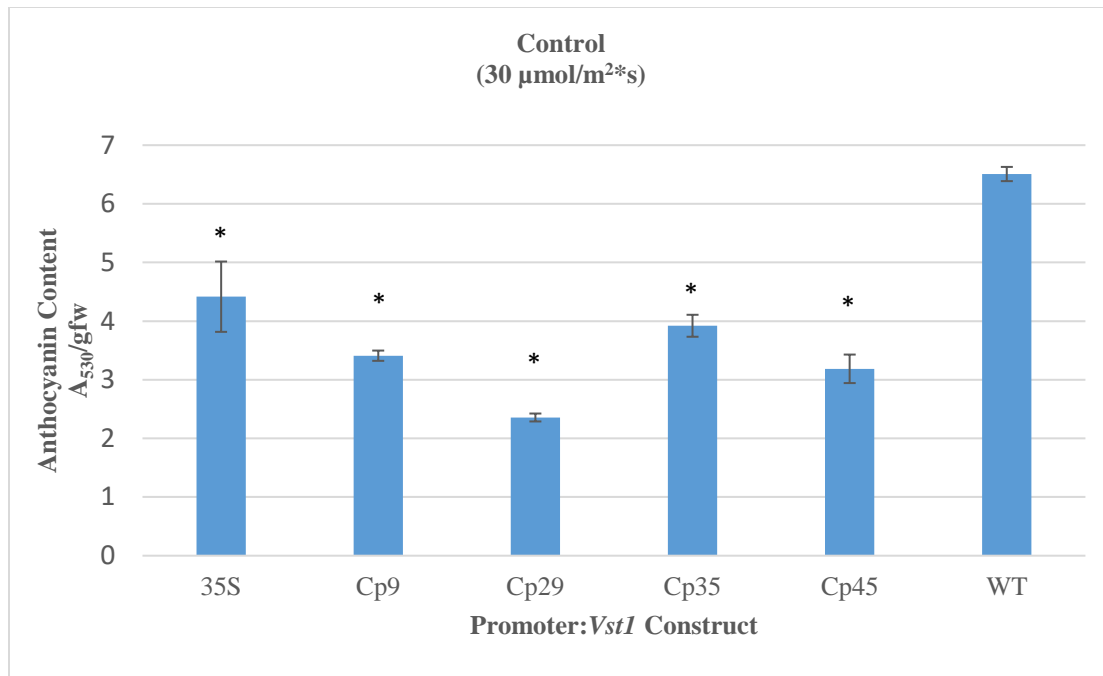


Figure 12: Anthocyanin content of representative lines of transgenic Arabidopsis containing each Cp promoter: *VstI* construct in control conditions (30 µmol/m²*s). Each construct consisted of 3 independent lines each, except *Cp45*, which had two. Error bars represent standard error, gfw = gram fresh weight. Asterisks denote significant reduction ($p < 0.05$) from WT as measured by a standard t-test.

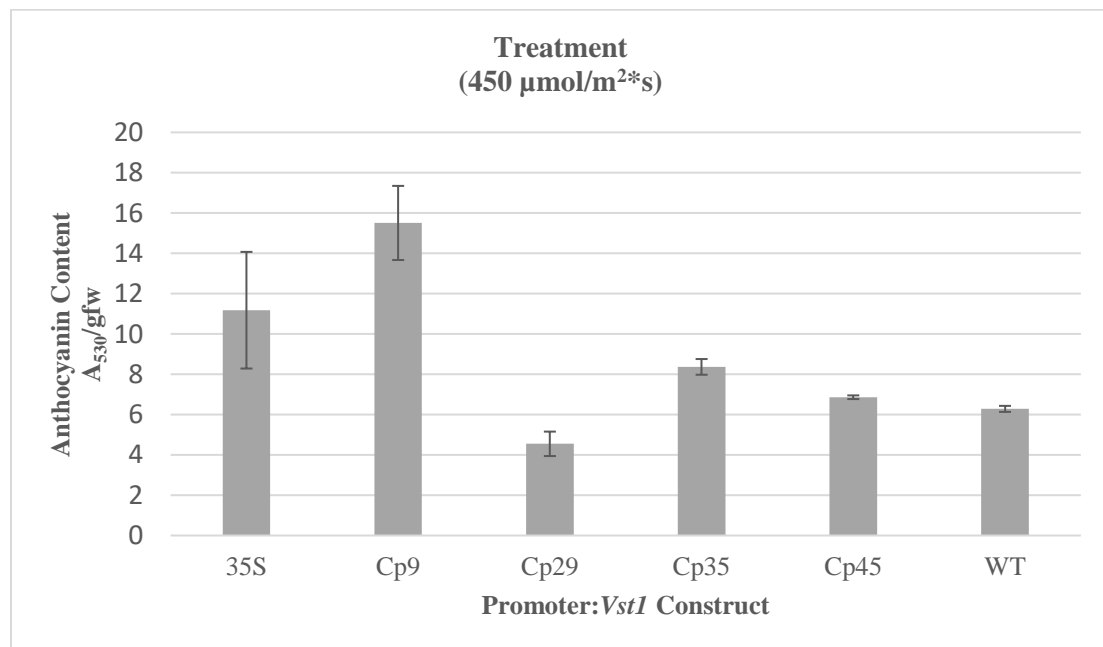


Figure 13: Anthocyanin content of representative lines of transgenic Arabidopsis containing each Cp promoter: *VstI* construct in treatment conditions (2 days at 450 µmol/m²*s). Each construct consisted of 3 independent lines each, except *Cp45*, which had two. Error bars represent standard error, gfw = gram fresh weight.

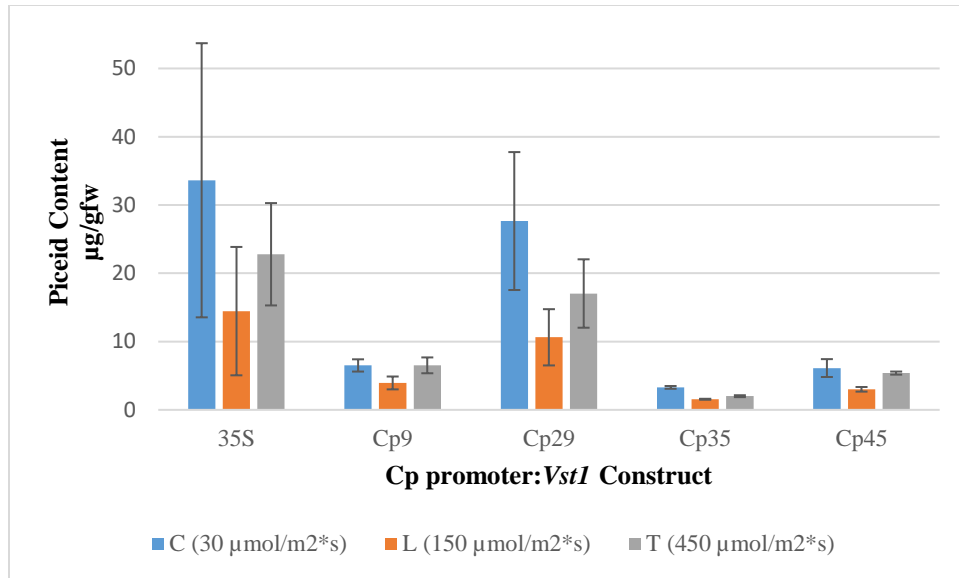


Figure 14. Piceid content of representative lines of transgenic *Arabidopsis* containing each Cp promoter: *VstI* construct in all three blue light treatment conditions. Each construct consisted of 3 independent lines each, except *Cp45*, which had two. Error bars represent standard error, gfw = gram fresh weight. Large error bars were due to high variability in piceid content of independent lines within promoter constructs.

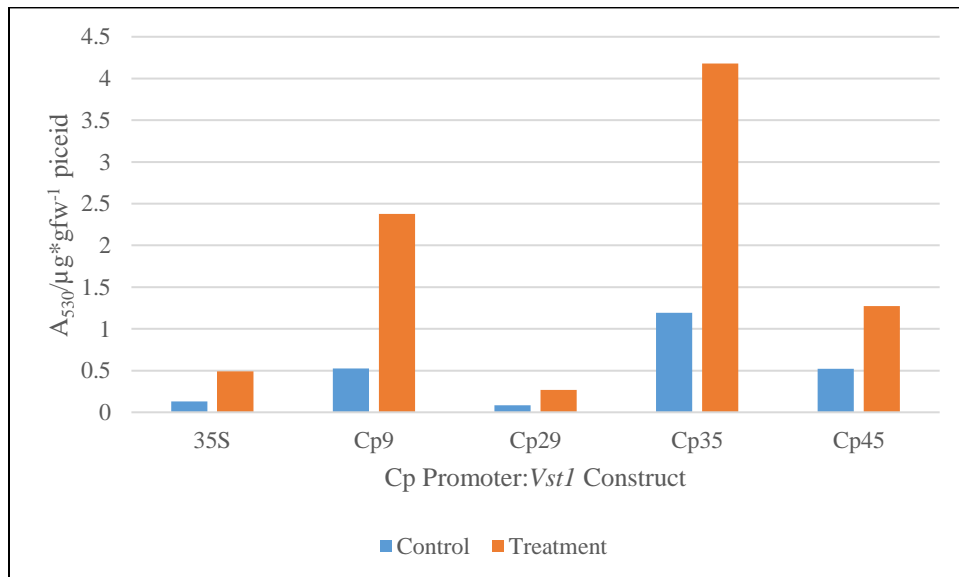


Figure 15. Ratio of anthocyanin content (A_{530}) to piceid content ($\mu\text{g/gfw}$) in each Cp promoter construct in control conditions ($30 \mu\text{mol/m}^2\text{s}$, fluorescent cool white light) or treatment conditions ($450 \mu\text{mol/m}^2\text{s}$ blue light). Each construct consisted of 3 independent lines each, except *Cp45*, which had two. Gfw = gram fresh weight.

Discussion

SA and MeJA Experiments

The effects of exogenous salicylic acid have been well documented as causing the host plant to activate defense responses and upregulate genes involved in defense. The results of the salicylic acid experiments show that expression of the *VstI* gene was not increased by treatment with SA in transgenic Arabidopsis lines containing any of the Cp promoter constructs. Since SA elicits a defense response in plants, a subset of genes that are involved in defense roles are upregulated as a result. Because the positive control gene *PR-1*, a quintessential indicator of the activation of the SA defense pathway, was upregulated in response to the treatment, salicylic acid-linked defense pathways in the Arabidopsis plants were likely activated. Additionally, considering the genes from which the Cp promoters were isolated from were upregulated during infection with *P. palmivora*, it is likely that these genes are involved in defense and that the regulatory elements contained in the promoters are recognized by at least some type of defense response signaling mechanisms in papaya. However, no regulation of the transgene was observed during the SA-induced defense response.

Two sets of explanations are apparent to describe this discrepancy. The first is simply that the Arabidopsis molecular defense response mechanisms do not recognize the regulatory elements contained in any of the papaya-isolated promoters. Because these promoters are non-native to Arabidopsis, there is no guarantee that any sort of transcriptional regulation can occur. *In silico* analyses of all four Cp promoter regions revealed at least one *cis*-acting element involved in salicylic acid responsiveness in three of the promoters, *Cp9*, *Cp35*, and *Cp45* (Carlos-Hilario, 2015). This would suggest that these three promoters would respond, at least to

some degree, to treatment with SA, which was not the case found here. All of the Cp promoter:*VstI* constructs are, however, expressed at a basal level, implying that a different transcriptional mechanism is responsible for regulation during a defense response, rather than simply increased activity by the same mechanism that is involved in basal expression.

The second possible explanation is that the Cp promoter:*VstI* constructs are regulated by defense pathways that are independent of the salicylic acid derived pathway. Indeed, other molecular defense pathways do exist, most notably the jasmonic acid/ethylene dependent pathway. Studies have shown that there are genes that are regulated by one pathway and not by the other (e.g. Thomma et al., 1998). However, preliminary experiments involving treatment with methyl jasmonate (MeJA) indicated no regulation of *VstI*, although these experiments are far from complete and were not pursued due to technical difficulties and time constraints (data not shown). As such, ruling this option out definitively would require additional experimentation.

It seems more likely that the former explanation, that Arabidopsis does not recognize the papaya promoters during a defense response, is the determining factor in this situation. If this is the case, then Arabidopsis is not an appropriate model to explore transcriptional regulation of the Cp promoter:*VstI* constructs in response to defense response elicitors. Now that at least some lines of transgenic papaya containing the same genetic constructs have been regenerated, it would be rewarding to investigate transcriptional regulation and piceid synthesis during a defense response in papaya, whether by defense response elicitors such as SA or MeJA, or by inoculation with *P. palmivora* itself.

Basal Piceid Content

Basal expression leading to constitutive production of piceid was one of the prerequisites of an ideal promoter in this study. Basal levels of piceid is thought to help ward off infection before the pathogen can get established. Therefore, to determine basal content, RP-HPLC was used to detect and quantify the piceid levels during normal growth conditions.

Basal piceid content in the *Cp35:Vst1* and the *Cp45:Vst1* lines were much lower than those of the *Cp29:Vst1* and the *35S:Vst1* lines, suggesting that the basal promoter activity of the *Cp35* and *Cp45* promoters is relatively weak. However, the *Cp45* promoter conferred a somewhat higher basal piceid content than that of the weak *Cp9* and *Cp35* promoters. The biochemical production of piceid in the Arabidopsis lines roughly matches the piceid content previously reported in the transgenic papaya somatic embryos in corresponding lines (Carlos-Hilario, 2015). Since high expression levels associated with the strong constitutive *35S* promoter have been correlated with adverse phenotypic effects such as male sterility and possible stunting (Fischer et al., 1997; Hilario et al., 2015), such high expression levels may not be desirable. The relatively lower expression of the *Cp45* promoter may avoid these effects (data not shown), though it still remains to be seen whether it provides enough basal piceid to ward off infection from *P. palmivora*.

Blue Light Experiments

Since the promoters used to drive the transgene are inducible promoters containing numerous predicted light responsive regulatory elements (Carlos-Hilario, 2015), these promoters may be regulated by different light conditions. Due to substrate competition between stilbene

synthase and chalcone synthase, any regulation of the transgene may affect the concentration of other secondary metabolites, such as anthocyanins or other flavonoids. On the other hand, any regulation of chalcone synthase may affect stilbenoid concentrations as well. Anthocyanin pigments are thought to help protect against UV damage and photoinhibition due to excess light energy, and a reduction of anthocyanin content may be detrimental to the health of the plant during UV or blue light exposure. In Arabidopsis plants under the control conditions (30 $\mu\text{mol}/\text{m}^2\cdot\text{s}$), anthocyanins content in each of the transgenic lines were significantly reduced as compared to the controls (Figure 12), possibly as a result of substrate competition. However, under high intensity blue light however, anthocyanin content was either similar or higher than WT controls (Figure 13). This suggests that though substrate competition may be present, the upregulation of flavonoid biosynthesis genes in response to blue light overrides stilbenoid production, leading to anthocyanin content that is at least that of the WT plants. Though there was a high degree of variability in piceid content in some of the Cp promoter:*VstI* constructs, the piceid content was not significantly different in response to exposure to blue light, as compared to the controls (Figure 13), suggesting that none of the papaya promoters are responsive to exposure to blue light in Arabidopsis. This seems to fit with the fact that anthocyanin content was not disrupted during high blue light conditions (450 $\mu\text{mol}/\text{m}^2\cdot\text{s}$), as there seems to be no concurrent upregulation of piceid biosynthesis to cause further competition for substrate molecules. Furthermore, the ratio of anthocyanin content to piceid content increased in each Cp promoter:*VstI* construct lines in the treatment conditions approximately 2.5 - 4.5 fold over their respective controls (Figure 15). This is very similar to the increase of anthocyanin content alone between treatment and control conditions (data not shown), supporting the theory that piceid levels are relatively unaffected by high blue light exposure, while anthocyanin content is

increased. This study has revealed some basic effects of substrate competition, and further study could elucidate the effects of competition and the mechanisms behind it more clearly.

Conclusion

This study used the relatively simple Arabidopsis model system to express the ectopic *VstI* gene driven by four different native papaya promoters, and characterize changes in expression and the associated piceid levels in response to various response elicitors. The SA experiments revealed that no expression level changes occur as a response to the defense response elicitor salicylic acid, and therefore it can be concluded that the *VstI* transgene is not part of the SA defense pathway when coupled to any of the native papaya promoters. Though the experiments involving the jasmonate pathway were not completed due to technical difficulties (i.e. failure of positive induction control) and time restraints, indications are that none of the promoters respond to elicitation from methyl jasmonate as well, though this would need to be confirmed in the future. Basal piceid content was measured in the *Cp35:VstI* and *Cp45:VstI* lines, with the *Cp35* lines containing a low amount of piceid and the *Cp45* lines containing an intermediate level of piceid as compared to the 35S positive control lines. In uninduced controls, significant reductions in anthocyanin levels in all transgenic Arabidopsis lines were found, as compared to the WT plants, suggesting that flavonoid levels are reduced due to substrate

competition in normal conditions. However, no significant reductions in anthocyanin content were found in blue light induced transgenic *Arabidopsis* lines, indicating that the induction of anthocyanins is largely unaffected by substrate competition. Though a high degree of variability was found, piceid content in the all of the transgenic *Arabidopsis* plants was not significantly different when induced with blue light as compared to uninduced controls. Based on piceid content from the transgenic *Arabidopsis* and the transgenic papaya somatic embryos (Carlos-Hilario, 2015), the *Cp45:Vst1* lines seem to be the most promising for future pathogen resistance studies.

REFERENCES

- Adrian, M., Jeandet, P., Veneau, J., Weston, L. A., Bessis, R. (1997). Biological activity of resveratrol, a stilbenic compound from grapevines, against *Botrytis cinerea*, the causal agent for gray mold. *J. Chem. Ecol.* 23: 1689-1702.
- Anekonda, T.S. (2006). Resveratrol – a boon for treating Alzheimer’s disease? *Brain Res. Rev.* 52: 316–326.
- Athar, M., Back, J.H., Tang, X., Kim, K.H., Kopelovich, L., Bickers, D.R., Kim, A.L. (2007). Resveratrol: a review of preclinical studies for human cancer protection. *Toxicol. Appl. Pharmacol.* 224: 274–283.
- Baur, J.A. and Sinclair, D.A. (2006). Therapeutic potential of resveratrol: the in vivo evidence. *Nat. Rev. Drug Discov.* 5: 493–506.
- Carletti, G., Nervo, G., Cattivelli, L. (2014). Flavonoids and melanins: a common strategy across two kingdoms. *Int. J. Biol. Sci.* 10(10): 1159-1170
- Carlos-Hilario, L. (2015). Metabolic engineering of *Arabidopsis thaliana* and *Carica papaya* using native papaya promoters to control stilbene synthase gene expression. Ph. D. dissertation, University of Hawai‘i at Mānoa.
- Chalal, M., Klinguer, A., Echairi, A., Meunier, P., Vervandier-Fasseur, D., Adrian, M. (2014). Antimicrobial activity of resveratrol analogues. *Molecules* 19: 7679–7688.
- Chen Z., Silva H., Klessig, D.F. (1993). Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. *Science* 262: 1883–1886.

- Christopher, D.A., Mullet, J.E. (1994). Separate photosensory pathways co-regulate blue light-ultraviolet A-activated *psbD-psbC* transcription and light-induced D2 and CP43 degradation in barley (*Hordeum vulgare*) chloroplasts. *Plant Phys.* 104:1119-1129.
- Darvill, A.G., Albersheim, P. (1984). Phytoalexins and their elicitors—a defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* 35:243–275.
- Delaney T.P., Uknes S., Vernooij B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E., Ryals, J. (1994). A central role of salicylic acid in plant disease resistance. *Science* 266: 1247–1250.
- Delaunoy, B., Cordelier, S., Conreux, A., Clement, C., & Jeandet, P. (2009). Molecular engineering of resveratrol in plants. *Plant Biotech. J.* 7(1): 2–12.
- Demmig-Adams, B., Adams, W.W. (1992). Photoprotection and other responses of plants to high light stress. *Annu. Rev. Plant Physiol. and Plant Mol. Biol.* 43: 599-626.
- Dercks, W., Creasy, L. L. (1989). The significance of stilbene phytoalexins in the *Plasmopara viticola*-grapevine interaction. *Physiol. Mol. Plant Pathol.* 34: 189-202.
- De Winaar, W. (1988). Clonal propagation of papaya *in vitro*. *Plant Cell Tiss. Org. Cult.* 12: 305-310.
- Dhekney, S.A, Kandel, R., Bergey, D.R., Sitther, V., Soorianathasundaram, K., Litz, R.E. (2016). Advances in papaya biotechnology. *Biocat. and Ag. Biotech.* 5: 133-142.
- Dodeman, V.L., Ducreux, G., Kreis, M. (1997). Zygotic embryogenesis *versus* somatic embryogenesis. *J. Exp. Bot.* 48: 1493–1509.

- Erwin, D.C., Ribeiro, O.K. (1996). *Phytophthora* Diseases Worldwide. St. Paul Minnesota: APS Press.
- Evans, E.A., Ballens, F.H. (2012). "An Overview of Global Papaya Production, Trade, and Consumption." Electronic Data Information Source, University of Florida. Retrieved from: <http://edis.ifas.ufl.edu/fe913>.
- Feys, B., Benedetti, C.E., Penfold, C.N. and Turner, J.G. (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. *Plant Cell* 6: 751–759.
- Fischer, R., Budde, I. and Hain, R. (1997). Stilbene synthase gene expression causes changes in flower colour and male sterility in tobacco. *Plant J.* 11: 489–498.
- Fitch, M.M.M., Manshardt, R.M., Gonsalves, D., Slightom, J.L., Sanford, J.C. (1992). Virus resistant papaya plants derived from tissues bombarded with the coat protein gene of papaya ringspot virus. *Bio/technology* 10(11): 1466-1472.
- Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43: 205–227.
- Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. and Ryals, J. (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* 8: 629-643.
- Greenberg, J.T. (1997). Programmed cell death in plant-pathogen interactions. *Annu. Rev. Plant Biol.* 48:525–45.

- Hain, R., Reif, H.J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P., Stöcker, R. and Stenzel, K. (1993). Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* 361: 153–156.
- Havaux, M., Kloppstech, K. (2001). The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in *Arabidopsis npq* and *tt* mutants. *Planta* 213: 953-966.
- Hilario, L.C., Porter, B.W., Zhu, Y.J., Christopher, D.A. (2014). Identification and characterization of papaya (*Carica papaya*, L.) promoters by heterologous expression as eGFP fusions in *Arabidopsis*. *Trop. Plant Biol.* 7: 85-99.
- Hilario, L.C., Shimshock, R., Ng, C., Bingham, J.P., Christopher, D.C. (2015). Screening *Carica papaya* native promoters driving stilbene synthase expression in *Arabidopsis thaliana* for resveratrol glucoside (piceid) synthesis. *Plant Biotech. Rep.* 9(5): 307-317.
- Hipskind, J.D., and Paiva, N.L. (2000). Constitutive accumulation of a resveratrol glucoside in transgenic alfalfa increases resistance to *Phoma medicaginis*. *Mol. Plant–Microbe Interact.* 13: 551–562.
- Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., Matsui, H. (2001). A large family of class III plant peroxidases. *Plant Cell Physiol.* 42: 462–468.
- Hwang, D., Lim, Y.H. (2015). Resveratrol antibacterial activity against *Escherichia coli* is mediated by Z-ring formation inhibition via suppression of FtsZ expression. *Scientific Reports* 5: 10029.
- Jain, S.M. (2001). Tissue culture-derived variation in crop improvement. *Euphytica* 118:153–166.

- Jeandet, P., Douillet-Breuil, A.C., Bessis, R., Debord, S., Sbaghi, M., Adrian, M. (2002).
Phytoalexins from the Vitaceae: biosynthesis, phytoalexin gene expression in transgenic
plants, antifungal activity, and metabolism. *J. Agric. Food Chem.* 50: 2731–2741.
- Jung, H.W., Hwang, B.K. (2007). The leucine-rich repeat (LRR) protein, CaLRR1, interacts with
the hypersensitive induced reaction (HIR) protein, CaHIR1, and suppresses cell death
induced by the CaHIR1 protein. *Mol. Plant Pathol.* 8: 503–514.
- Kim, Y.J., Kim, D.G., Lee, S.H., Lee, I. (2006). Wound-induced expression of the ferulate 5-
hydroxylase gene in *Camptotheca acuminata*. *Biochimica et Biophysica Acta* 1760: 182–
190.
- King, R.E., Bomser, J.A. and Min, D.B. (2006) Bioactivity of resveratrol. *Compr. Rev. Food Sci.*
Food Safety, 5: 65–70.
- Koes, R.E., Quattrocchio, F., Mol, J.N.M.. (1994). The flavonoid biosynthetic pathway in plants:
function and evolution. *BioEssays* 16: 123–132.
- Komamine, A., Kawahara, R., Matsumoto, M., Sunabori, S., Toya, T., Fujiwara, A., Tsukahara,
M., Smith, J., Ito, M., Fukuda, H., Nomura, K., Fujimura, T. (1992). Mechanisms of
somatic embryogenesis in cell cultures: physiology, biochemistry, and molecular biology.
In Vitro Cell Dev. Biol. Plant 28:11–14.
- Kong, J.M., Chia, L.S., Goh, N.K., Chia, T.F., Brouillard, R. (2003). Analysis and biological
activities of anthocyanins. *Phytochem.* 64(5): 923–933.

- Kubasek, W.L., Shirley, B.W., Mckillop, A., Goodman, H.M., Briggs, W., Ausubel, F.M. (1992). Regulation of flavonoid biosynthetic genes in germinating *Arabidopsis* seedlings. *The Plant Cell*. 4: 1229-1236.
- Lamb, C., Dixon, R.A. (1997). The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48: 251–275.
- Lamour, K.H., Win, J., Kamoun, S. (2007). Oomycete genomics: new insights and future directions. *FEMS Microbiol. Lett.* 274: 1–8.
- Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T. and Ryals, J. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *Plant J.* 10: 71–82.
- Leckband, G., and Lörz, H. (1998). Transformation and expression of a stilbene synthase gene of *Vitis vinifera* L. in barley and wheat for increased fungal resistance. *Theor. Appl. Genet.* 96: 1004–1012.
- Lee, J., Lee, D.G. (2015). Novel Antifungal Mechanism of Resveratrol: Apoptosis Inducer in *Candida albicans*. *Curr. Microbiol.* 70: 383–389.
- Leljak-Levanic, D., Bauer, N., Mihaljevic, S., Jelaska, S. (2004). Changes in DNA methylation during somatic embryogenesis in *Cucurbita pepo* L. *Plant Cell Rep.* 23:120–127.
- Liswidowati, F., Melchior, F., Hohmann, F., Schwer, B., Kindl, H. (1991). Induction of stilbene synthase by *Botrytis cinerea* in cultured grapevine cells. *Planta* 183:307-314.
- Liszakay, A., Kenk, B., Schopfer, P. (2003). Evidence for the involvement of cell wall peroxidase in the generation of hydroxyl radicals mediating extension growth. *Planta* 217:658–667.

- Liu, Z.Y., Zhuang, C.X., Sheng, S.J. Shao, L., Zhao, W., Zhao, S. (2011). Overexpression of a resveratrol synthase gene (*PcRs*) from *Polygonum cuspidatum* in transgenic *Arabidopsis* causes the accumulation of *trans*-piceid with antifungal activity. *Plant Cell Rep.* 30: 2027–2036.
- LoSchiavo, F., Pitto, L., Giuliano, G., Torti, G., Nuti-Ronchi, V., Marazziti, D., Veraga, R., Orselli, S., Terzi, M. (1989). DNA methylation of embryogenic carrot cell cultures and its variations as caused by maturation, differentiation, hormones and hypomethylation drugs. *Theor. App. Genet.* 77: 325.
- Manshardt, R.M. (1992). Papaya. In: Hammerschlag, F.A., Litz, R.E. (eds.) *Biotechnology of Perennial Fruit Crops*. CAB International, Wallingford, UK pp. 489-511.
- Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A., Chapple, C. (1998). Lignin monomer composition is determined by the expression of a cytochrome P450-dependent monooxygenase in *Arabidopsis*. *Proc. of the Natl. Acad. of Sci. of the United States of America.* 95:6619–23.
- Ming, R., Hou, S., Feng, Y., Yu, Q., Dionne-Laporte, A., Saw, J.H., Senin, P., Wang, W., Ly, B.V., Lewis, K.L., Salzberg, S.L., Feng, L., Jones, M.R., Skelton, R.L., Murray, J.E., Chen, C., Qian, W., Shen, J., Du, P., Eustice, M., Tong, E., Tang, H., Lyons, E., Paull, R.E., Michael, T.P., Wall, K., Rice, D., Albert, H., Wang, M.L., Zhu, Y.J., Schatz, M., Nagarajan, N., Agbayani, R., Guan, P., Blas, A., Wai, C.M., Ackerman, C.M., Ren, Y., Liu, C., Wang, J., Wang, J., Na, J.K., Shakhov, E.V., Haas, B., Thimmapuram, J., Nelson, D., Wang, X., Bowers, J.E., Gschwend, A.R., Delcher, A.L., Singh, R., Suzuki, J.Y., Tripathi, S., Neupane, K., Wei, H., Irikura, B., Paidi, M., Jiang, N., Zhang, W.,

- Presting, G., Windsor, A., Navajas-Pe´rez, R., Torres, M.J., Feltus, A.F., Porter, B., Li, Y., Burroughs, A.M., Luo, M.C., Liu, L., Christopher, D.A., Mount, S.M., Moore, P.H., Sugimura, T., Jiang, J., Schuler, M.A., Friedman, V., Mitchell-Olds, T., Shippen, D.E., Depamphilis, C.W., Palmer, J.D., Freeling, M., Paterson, A.H., Gonsalves, D., Wang, L., Alam, M. (2008). The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature* 452: 991–996.
- Murashige, T. and Skoog, F. 1962, A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiol. Plant* 15:493–497.
- National Agriculture Statistical Service (NASS). “2014 State Agriculture Overview.” Retrieved from: http://www.nass.usda.gov/Quick_Stats/Ag_Overview/stateOverview.php?state=HAWAII.
- Nissen, S., Sutter, E. (1990). Stability of IAA and IBA in nutrient medium to several tissue culture procedures. *HortScience* 25(7): 800-802.
- Palva, T.K., Hurtig, M., Saindrenan, P., Palva, E.T. (1994). Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. *Mol. Plant Microbe Interact.* 7:356 – 363.
- Parage, C., Tavares, R., Réty, S., Baltenweck-Guyot, R., Poutaraud, A., Renault, L., Heintz, D., Lugan, R., Marais, G.A.B., Aubourg, S., Hugueney, P. (2012). Structural, functional, and evolutionary analysis of the unusually large stilbene synthase gene family in grapevine. *Plant Phys.* 160 (3): 1407-1419.

- Passardi, F., Longet, D., Penel, C., Dunand, C. (2004). The plant peroxidase multigenic family in rice and its evolution in green plants. *Phytochem.* 65:1879–1893.
- Passardi, F., Cosio, C., Penel, C., Dunand, C., (2005). Peroxidases have more functions than a Swiss army knife. *Plant Cell Rep.* 24: 255-265.
- Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Métraux, J.P., Manners, J.M., Broekaert, W.F. (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. *Plant Cell.* 8(12): 2309-23.
- Persley, D.M., Ploetz, R.C. (2003). Diseases of Papaya. In: Ploetz, R.C. (ed.), *Diseases of Tropical and Subtropical Fruit Crops*. Wallingford, Oxon, GBR: CABI Publishing, pp. 376-413. Retrieved from <http://www.ebrary.com>.
- Pezet, R., Perret, C., Jean-Denis, J.B., Tabacchi, R., Gindro, K., Viret, O. (2003). δ -viniferin, a resveratrol dehydrodimer: one of the major stilbenes synthesized by stressed grapevine leaves. *J. Agric. Food Chem.* 51: 5488–5492.
- Porchewski, P., Specht, V., Stubner, S., Kindl, H. (2001). A novel tetratricopeptide repeat-containing J-protein localized in a plasma membrane-bound protein complex of the phytopathogenic oomycete *Phytophthora megasperma*. *Eur. J. Cell Biol.* 80: 527-538.
- Porter, B.W., Aizawa, K.S., Zhu, Y.J., Christopher, D.A. (2008). Differentially expressed and new non-protein-coding genes from a *Carica papaya* root transcriptome survey. *Plant Science* 174: 38-50.

- Porter B.W., Zhu, Y.J., Christopher, D.A. (2009). *Carica papaya* genes regulated by *Phytophthora palmivora*: A new system for comparative genomics. *Trop. Plant Biol.* 2: 84–97.
- Porter B.W., Christopher D.A., Zhu Y.J., (2013). Genomics of Papaya Disease Resistance. In: Ming, R. Moore, P. H. (eds), *Genetics and Genomics of Papaya* (series on Plant Genetics and Genomics: Crops and Models), Springer New York, pp. 277-307.
- Rabino, I., Mancinelli, A.L. (1986). Light, temperature, and anthocyanin production. *Plant Phys.* 81: 922-924.
- Ruegger, M., Meyer, K., Cusumano, J.C., Chapple, C. (1999). Regulation of ferulate-5-hydroxylase expression in *Arabidopsis* in the context of sinapate ester biosynthesis. *Plant Physiol.* 119(1): 101–110.
- Sasaki, Y., Asamizu, E., Shibata, D., Nakamura, Y., Kaneko, T., Awai, K., Amagai, M., Kuwata, C., Tsugane, T., Masuda, T., Shimada, H., Takmiya, X., Ohta, H., Tabata, S. (2001). Monitoring of methyl jasmonate-responsive genes in *Arabidopsis* by cDNA macroarray: self-activation of jasmonic acid biosynthesis and crosstalk with other phytohormone signaling pathways. *DNA Res.* 8(4): 153–61.
- Schröder, G., Brown, J.W., Schröder, J. (1988). Molecular analysis of resveratrol synthase. *Eur. J. Biochem.* 172(1): 161–169.
- Schröder, J., Schröder, G. (1990). Stilbene and chalcone synthases: related enzymes with key functions in plant-specific pathways. *Z. Naturforsch* 45: 1-8.

- Senaratna, T., Touchell, D., Bunn, T., and Dixon, K. (2000) Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. *Plant Growth Regul.* 30: 157–161.
- Serazetdinova, L., Oldach, K. and Lörz, H. (2005). Expression of transgenic stilbene synthases in wheat causes the accumulation of unknown stilbene derivatives with antifungal activity. *J. Plant Physiol.* 162: 985–1002.
- Smillie, R.M., Hetherington, S.E. (1999). Photoabatement by anthocyanin shields photosynthetic systems from light stress. *Photosynthetica* 36: 451-463.
- Smith, J.A., Hammerschmidt, R., Fulbright, D.W. (1991). Rapid induction of systemic resistance in cucumber by *Pseudomonas syringae* pv. *syringae*. *Physiol. Mol. Plant Pathol.* 38: 223-235.
- Sobolev, V. S., Khan, S. I., Tabanca, N., Wedge, D. E., Manly, S. P., Cutler, S. J., Coy, M. R., Becnel, J. J., Neff, S. A., Gloer, J. B. (2011). Biological activity of peanut (*Arachis hypogaea*) phytoalexins and selected natural and synthetic stilbenoids. *J. Agric. Food Chem.* 59: 1673–1682.
- Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., van Loon, L.C., Dong, X.N., Pieterse, C.M.J. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *Plant Cell* 15: 760–770.
- Staswick, P.E., Tiriyaki, I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. *Plant Cell* 16: 2117–27.

- Storey, W.B. (1976). Papaya. In: Simmonds, N.W. (ed.) *Evolution of Crop Plants*. Longman, London pp. 21-24.
- Subramanian, M., Goswami, M., Chakraborty, S., Jawali, N. (2014). Resveratrol induced inhibition of *Escherichia coli* proceeds via membrane oxidation and independent of diffusible reactive oxygen species generation. *Redox Biol.* 2: 865–872.
- Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci.* 95: 15107–15111.
- Tropf, S., Lanz, T., Rensing, S.A., Schroder, J., Schroder, G. (1994). Evidence that stilbene synthases have developed from chalcone synthases several times in the course of evolution, *J. Mol. Evol.* 38: 610–618.
- Tsao, R. (2010). Chemistry and biochemistry of dietary polyphenols. *Nutrients* 2(12): 1231-1246.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., Ryals, J. (1992). Acquired resistance in *Arabidopsis*. *Plant Cell* 4(6): 645-656.
- Uknes, S., Winter, A., Delaney, T., Potter, S., Ward, E. and Ryals, J. (1993). Biological induction of systemic acquired resistance in *Arabidopsis*. *Mol. Plant-Microbe Interact.* 6: 692-698.
- van Loon, L.C., Rep, M., Pieterse, C.M.J. (2006). Significance of inducible defense-related proteins in infected plants. *Annu. Rev. of Phytopath.* 44: 135–162.

- von Arnold, S., Sabala, I., Bozhkov, P., Kyachok, J., Filonova, L. (2002). Developmental pathways of somatic embryogenesis. *Plant Cell Tiss. Org. Cult.* 69: 233–249.
- Wu, K., Zeng, S., Chen, Z., Duan, J. (2012). *In vitro* propagation of hermaphroditic *Carica papaya* cv. Meizhonghong. *Pak. J. Bot.* 44(5): 1669-1676.
- Yang, J.S., Ye, C.A. (1992). Plant regeneration from petioles of *in vitro* regenerated papaya (*Carica papaya* L.) shoots. *Bot. Bull. Acad. Sin.* 33:375-381.
- Zhu, Y.J., Agbayani, R., Jackson, M.C., Moore, P. (2004). Expression of the grapevine stilbene synthase gene *VST1* in papaya provides increased resistance against diseases caused by *Phytophthora palmivora*. *Planta* 220: 241-250.